



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent No. : 5,639,940  
Patentee : Ian Garner, Michael A. Dalrymple\*, Donna E. Prunkard and Donald C. Foster  
Assignee : Pharmaceutical Proteins, Ltd. and ZymoGenetics, Inc.  
Issued : June 17, 1997  
Application : 08/206,176  
Filed : March 3, 1994  
For : PRODUCTION OF FIBRINOGEN IN TRANSGENIC ANIMALS

New York, New York  
January 15, 1999

Hon. Assistant Commissioner for Patents  
Washington, D.C. 20231

PETITION TO CORRECT INVENTORSHIP

Sir:

The patentees and the assignees of record of the above-identified Garner patent ("the '940 patent") petition to correct inventorship by deleting Ian Garner and Michael A. Dalrymple as named inventors. 37 C.F.R. § 1.324. After the correction, co-inventors Donna E. Prunkard and Donald C. Foster will remain as the joint inventors of the '940 patent.

Supporting papers filed herewith are *Declaration Under 37 C.F.R.*

*§ 1.324(b)(1) and (b)(2) of Ian Garner, Michael A. Dalrymple, Donna E. Prunkard and*

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\* Dr. Dalrymple's middle initial was erroneously printed as "L" in the '940 patent.

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*Donald C. Foster* (Exhibit 1), *Declaration of Gary E. Parker Under 37 C.F.R. § 1.324(a)* (Exhibit 2), and two *Consents of Assignee to Correct Inventorship* (Exhibits 3 and 4). The *Consents* are executed by the assignees of record, Pharmaceutical Proteins, Ltd. and ZymoGenetics, Inc., respectively.

Because the '940 patent is now part of *Garner v. Velandar*, Interference No. 104,242, this petition and its supporting papers were also filed in the interference.

As described in detail below, Donna E. Prunkard and Donald C. Foster are the true inventors of the subject matter claimed in the '940 patent. Ian Garner and Michael A. Dalrymple merely assisted Donna E. Prunkard and Donald C. Foster in reducing the claimed invention to practice, and did not make any inventive contribution to the invention.

A. The Legal Standard

"Conception" is the formation in the mind of the inventor of a definite idea of the "complete and operative invention." *See, e.g., Burroughs Welcome Co. v. Barr Laboratories Inc.*, 40 F.3d 1223, 1228, 32 USPQ.2d 1915, 1919 (Fed. Cir. 1994).

"Reduction to practice" is the actual performance or carrying out of the invention. One who conceives is an inventor. *Id.* One who reduces to practice an already conceived invention is not an inventor. *Id.*

A definite idea of the complete and operative invention, i.e., conception, is had when a skilled worker could put the idea into practice without inventive skill or undue experimentation. *See, e.g., Sewall v. Walters*, 21 F.3d 411, 414-415, 30 USPQ2d 1356, 1359 (Fed. Cir. 1994):

Conception is complete when one of ordinary skill in the art

could [practice the invention] without unduly extensive research or experimentation (emphasis added).

Sewall alleged that he aided Walters in conceiving the invention and should therefore be named as a co-inventor. The Court held, however, that when Walters communicated his inventive ideas to Sewall, no more than ordinary skill would have been needed to reduce to practice Walters' proposed invention. As a result, the Court found Walters to be the sole inventor of the claimed invention. *See also, Summers v. Vogel*, 332 F.2d 810, 816, 141 USPQ 816, 820 (CCPA 1964) (senior party in interference had conceived the instrument of the count because construction of the claimed instrument on the basis of that conception "required only the exercise of the ordinary skill of the art").

The same legal standards apply to joint inventorship. Each joint inventor must "make some contribution to the inventive thought and to the final result." *Monsanto v. Kamp*, 269 F. Supp. 818, 824, 154 USPQ 259, 262 (D. D.C. 1967). *See also Williams Serv. Group Inc. v. O.B. Cannon and Son Inc.*, 33 USPQ2d 1705, 1727 (E.D. Pa. 1994). Further, a complete and operative conception which one inventor makes "before commencement of the collaborative effort never can be treated as the conception of a joint invention or as a joint invention." *General Motors Corp. v. Toyota Motors Co. Ltd.*, 467 F. Supp. 1142, 1163, 205 USPQ 158, 179 (S.D. Ohio 1979).

B. Donna Prunkard and Donald Foster Are the Joint Inventors

Donna Prunkard and Donald Foster jointly conceived the production of biocompetent fibrinogen in the milk of transgenic animals and of transgenic animals capable of producing such fibrinogen (Exhibit 1, ¶ 2). They made that conception before they had any discussions or began working with Ian Garner and Michael A. Dalrymple in connection with the transgenic production of fibrinogen (Exhibit 1, ¶ 2).

At the time of their conception, biocompetent human fibrinogen had been produced in mammalian cell cultures using cDNA constructs encoding and capable of expressing, respectively, the A $\alpha$ , B $\beta$  and  $\gamma$  chains of fibrinogen. *See, e.g., Roy et al., J. Biol. Chem.*, 266, pp. 4758-4763 (1991) (three different cDNA constructs each encoding a fibrinogen A $\alpha$ , B $\beta$  or  $\gamma$  chain were co-transfected and expressed in cultured monkey kidney cells and cultured human hepatocytes) (Exhibit 5); Farrell et al., *Biochemistry*, 30, pp. 9414-9420 (1991) (three different cDNA constructs each encoding a fibrinogen A $\alpha$ , B $\beta$  or  $\gamma$  chain were co-transfected and expressed in cultured baby hamster kidney cells) (Exhibit 6).

Further, at the time Prunkard and Foster conceived their invention, mixtures of multiple DNA constructs had been used to establish transgenic animals that contained in their genomes all the injected constructs. *See, e.g., Burdon et al., J. Biol. Chem.*, 266, pp. 6909-6914 (1991) (three different transgenes, each representing an allele of the mouse whey acidic protein gene, were co-injected into mouse zygotes which developed into mice that carried all three transgenes) (Exhibit 8). Transgenic animals established in this manner were capable of producing the desired heterologous multi-subunit protein in a targeted tissue such as the mammary gland. *See, e.g., Greenberg et al., Proc. Natl. Acad. Sci.*, 88, pp. 8327-8331 (1991) (co-injections of two separate transgenes encoding the alpha and beta subunits, respectively, of the heterodimeric bovine follicle-stimulating hormone gave rise to transgenic mice that produced in their milk the biologically active hormone) (Exhibit 7); Storb et al., *J. Exp. Med.*, 164, pp. 627-641 (1986) (mouse zygotes co-injected with rearranged mu and kappa genes developed into transgenic mice that produced the desired assembled, tetrameric antibodies in their B cells)



(Exhibit 9); and Behringer et al., Science, 245, pp. 971-973 (1989) (co-injection of DNAs encoding human  $\alpha$  and  $\beta$ -globin into mouse zygotes gave rise to transgenic mice that produced assembled, tetrameric human hemoglobin in the targeted tissue (Exhibit 13).

Simply put, at the time of the conception of Prunkard and Foster, the state of the recombinant fibrinogen and transgenic arts was sufficiently high for skilled workers in those arts to carry out, without inventive effort or undue experimentation, all the construction and procedural steps necessary to produce a transgenic animal that contained and expressed transgenes encoding each of the three fibrinogen chains in mammary tissues. All that remained to be accomplished after Prunkard and Foster conceived their invention and communicated that conception to Garner and Dalrymple was reduction to practice. Given the state of the art, that required no "unduly extensive research or experimentation" (*supra*, p. 3).

Ian Garner and Michael Dalrymple merely assisted Donna Prunkard and Donald Foster in reducing to practice the already conceived invention (Exhibit 1, ¶ 3). They provided the cloning vector into which Ms. Prunkard separately inserted the genomic DNA sequences coding for the A $\alpha$ , B $\beta$  and  $\gamma$  chains of fibrinogen (Exhibit 1, ¶ 3). That cloning vector is not claimed in the '940 patent or the application from which it issued (Exhibit 1, ¶ 3). In fact, a similar vector had already been described in the art before that cloning vector was provided to Prunkard and Foster. *See, e.g.*, WO 90/05188 (Exhibit 1, ¶ 3; Exhibit 10).

Ian Garner and Michael Dalrymple also assisted Donna Prunkard and Donald Foster in reducing to practice the already conceived invention by (1) supervising the injection into mouse zygotes of mixtures of DNA fragments prepared from the three

fibrinogen DNA constructs that Donna Prunkard and Donald Foster had made and provided to them for just that purpose, and (2) supervising the making of transgenic animals from these injected zygotes (Exhibit 1, ¶ 3). Such injections and procedures were not inventive. As discussed *supra* pp. 4-5, at the time of the Prunkard and Foster conception, mixtures of multiple DNA constructs had already been used to transfect mammalian cells and to inject mammalian zygotes, and transgenic animals that produced a desired heterologous multi-subunit protein in a targeted tissue (e.g., the mammary gland) had been produced from such zygotes (Exhibit 1, ¶ 3).

In sum, Ian Garner and Michael Dalrymple did not make an inventive contribution to the claims of the '940 patent. Conception of the invention by Donna Prunkard and Donald Foster was complete before Garner and Dalrymple became their collaborators in the production of biocompetent fibrinogen in the milk of transgenic animals. At best, Garner and Dalrymple assisted the true joint inventors of the claimed subject matter, Donna Prunkard and Donald Foster, in reducing the already conceived and completed invention to practice.

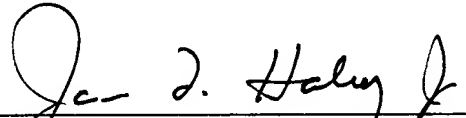
The error in naming Ian Garner and Michael Dalrymple as joint inventors on the Garner application arose without deceptive intent (Exhibit 1, ¶ 4; Exhibit 2, ¶ 7; Exhibits 11 and 12). The original designation looked to those individuals who had made intellectual contributions to either the conception or reduction to practice of the production of biocompetent human fibrinogen in the milk of transgenic mice (Exhibit 1, ¶ 4; Exhibit 2, ¶ 7; Exhibits 11 and 12). It did not separately consider those involved in conceiving the claimed invention as opposed to those involved merely in its actual reduction to practice. This error was not discovered until patentees and their counsel

reviewed, in connection with the *Garner v. Velander* interference, the Garner application, the '940 patent, and the work underlying the inventions claimed in the application and patent (Exhibit 1, ¶ 5; Exhibit 2, ¶ 8).

In view of the foregoing, patentees request that this Petition be granted and the inventorship be amended.

The Commissioner is hereby authorized to charge the \$130 petition fee (37 C.F.R. § 1.20(b)) and any additional fee due, or to credit any overpayment, in connection with this Petition, to Deposit Account No. 06-1075. A duplicate copy of this Petition is enclosed herewith.

Respectfully submitted,



James F. Haley, Jr., Reg. No. 27,794

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Gary E. Parker, Reg. No. 31,648

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Seattle, WA 98102

Exhibit 1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent No. : 5,639,940  
Patentee : Ian Garner, Michael A. Dalrymple\*, Donna E. Prunkard and Donald C. Foster  
Assignee : Pharmaceutical Proteins, Ltd. and ZymoGenetics, Inc.  
Issued : June 17, 1997  
Application : 08/206,176  
Filed : March 3, 1994  
For : PRODUCTION OF FIBRINOGEN IN TRANSGENIC ANIMALS

New York, New York  
January 15, 1999

Hon. Assistant Commissioner for Patents  
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.324(b)(1) AND (b)(2)  
OF IAN GARNER, MICHAEL A. DALRYMPLE,  
DONNA E. PRUNKARD AND DONALD C. FOSTER

Sir:

We, IAN GARNER, MICHAEL A. DALRYMPLE, DONNA E. PRUNKARD, and DONALD C. FOSTER, hereby declare that:

1. We are the named inventors of the above-identified patent and application from which it issued, and make this declaration in support of the accompanying *Garner's Motion to Correct Inventorship* and *Garner's Petition to Correct Inventorship*.

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\* Dr. Dalrymple's middle initial was erroneously printed as "L" in the '940 patent.

2. As to Donna E. Prunkard and Donald C. Foster, we conceived the production of biocompetent fibrinogen in the milk of transgenic animals and of the transgenic animals capable of producing such fibrinogen. This conception occurred before either of us had any discussions or began working with Ian Garner and Michael A. Dalrymple in connection with the production of fibrinogen in the milk of transgenic animals. We believe that at the time of our conception, all that remained to be accomplished was the actual reduction to practice of the conceived invention. We do not believe that such reduction to practice required any inventive skill or undue experimentation.

3. As to Ian Garner and Michael A. Dalrymple, we did not aid Donna E. Prunkard and Donald C. Foster in their conception of making biocompetent fibrinogen in the milk of transgenic animals or of transgenic animals capable of producing such fibrinogen. We did not have any discussions or begin our collaboration with them in regard to the production of fibrinogen in the milk of transgenic animals until they had completed their conception.

We assisted Donna E. Prunkard and Donald C. Foster in making transgenic mice that produced biocompetent human fibrinogen in their milk. We provided the cloning vector into which Ms. Prunkard separately inserted the genomic DNA sequences coding for the  $\text{A}\alpha$ ,  $\text{B}\beta$  and  $\gamma$  chains of human fibrinogen. That cloning vector is not claimed in the '940 patent. Before we provided that cloning vector to Donna Prunkard and Donald Foster, a similar vector had been described in international published patent application WO 90/05188 (Garner Exhibit 10).

We also assisted Donna Prunkard and Donald Foster in making transgenic

mice by supervising: (1) the injection into mouse oocytes of mixtures of DNA fragments prepared from the three fibrinogen DNA constructs that Ms. Prunkard had made and provided to us for that purpose, and (2) the making of transgenic animals from these oocytes. Such procedures required no inventive effort or undue experimentation on our part.

In fact, at this time, biocompetent human fibrinogen had been produced in cultured mammalian cells stably transfected with three cDNA constructs that encoded and expressed, respectively, the  $\text{A}\alpha$ ,  $\text{B}\beta$  and  $\gamma$  chain of fibrinogen. See, e.g., Roy et al., J. Biol. Chem., 266, pp. 4758-4763 (1991) (three different cDNA constructs each encoding a fibrinogen  $\text{A}\alpha$ ,  $\text{B}\beta$  or  $\gamma$  chain were co-transfected and expressed in cultured monkey kidney cells and cultured human hepatocytes) (Garner Exhibit 5); Farrell et al., Biochemistry, 30, pp. 9414-9420 (1991) (three different cDNA constructs each encoding a fibrinogen  $\text{A}\alpha$ ,  $\text{B}\beta$  or  $\gamma$  chain were co-transfected and expressed in cultured baby hamster kidney cells) (Garner Exhibit 6).

Further, by this time, mixtures of multiple DNA constructs had been used to establish transgenic animals that contained in their genomes all the injected constructs. Transgenic animals established in this manner were capable of producing a desired heterologous multi-subunit protein in a targeted tissue, such as the mammary gland. See, e.g., Greenberg et al., Proc. Natl. Acad. Sci., 88, pp. 8327-8331 (1991) (co-injections of the two separate transgenes encoding the alpha and beta subunits, respectively, of the heterodimeric bovine follicle-stimulating hormone gave rise to transgenic mice that produced in their milk the biologically active hormone) (Garner Exhibit 7); Burdon et al., J. Biol. Chem., 266, pp. 6909-6914 (1991) (three different transgenes, each representing

an allele of the mouse whey acidic protein gene, were co-injected into mouse oocytes which developed into mice that carried and expressed in their mammary glands all three transgenes) (Garner Exhibit 8); Storb et al., J. Exp. Med., 164, pp. 627- (1986) (co-injection of rearranged mu and k genes develop transgenic mice that produce the desired assembled antibodies in their B cells) (Garner Exhibit 9).

We believe that we made no inventive contribution to the subject matter claimed in the '940 patent or the application from which it issued.

4. We believe and understand that the error in naming Ian Garner and Michael A. Dalrymple as co-inventors on the Garner application, as filed, arose without deceptive intent. We understand that the error resulted from naming on the application those who had made intellectual contributions to either the conception or reduction to practice of the production of biocompetent fibrinogen in the milk of transgenic mice. We understand that the original designation of inventorship did not separately consider those involved in conceiving the invention as opposed to those involved merely in its actual reduction to practice.

5. We believe and understand that the error in inventorship was not discovered until we and our counsel reviewed, in connection with *Garner v. Velander*, Interference No. 104,242, the '940 patent in interference, the application from which the patent issued, and the work underlying the inventions claimed in the application and patent.

6. We hereby declare further that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that

willful false statements and the like so made are punishable by fine, or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the above-identified patent.

15TH January 1999  
Date

Ian Garner  
Ian Garner

15/1/99  
Date

M. Dalrymple  
Michael A. Dalrymple

1-13-99  
Date

Donna E. Prunkard  
Donna E. Prunkard

1-13-99  
Date

Donald C. Foster  
Donald C. Foster



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent No. : 5,639,940  
Patentee : Ian Garner, Michael A. Dalrymple\*, Donna E. Prunkard and Donald C. Foster  
Assignee : Pharmaceutical Proteins, Ltd. and ZymoGenetics, Inc.  
Issued : June 17, 1997  
Application : 08/206,176  
Filed : March 3, 1994  
For : PRODUCTION OF FIBRINOGEN IN TRANSGENIC ANIMALS

DECLARATION OF GARY E. PARKER  
UNDER 37 C.F.R. § 1.324(a)

Sir:

I, GARY E. PARKER, hereby declare that:

1. I make this declaration in support of the accompanying *Garner's Motion to Correct Inventorship* and *Garner's Petition to Correct Inventorship*.
2. I am Principle Patent Agent at ZymoGenetics, Inc., Seattle, Washington. In March 1994, I was the Manager of the Patent Department at ZymoGenetics. I am registered to practice before the United States Patent and Trademark Office. I am not an attorney.

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\* Dr. Dalrymple's middle initial was erroneously printed as "L" in the '940 patent.

3. I prepared and filed the '176 application that issued as the above-identified '940 patent. Before filing the application, I considered the inventorship of the claims presented.

4. In my consideration of inventorship, I investigated who had made an intellectual contribution to either the conception or reduction to practice of the claimed subject matter. See my pre-filing letter to Andy Carver, an embryologist at Pharmaceutical Proteins, Ltd. (Garner Exhibit 11; partially expurgated):

Under U.S. law, an inventor is one who made an inventive contribution to the claimed subject matter. Although the definition sounds circular, in general an inventor is one who made an intellectual contribution to the conception or reduction to practice of the invention, that is one who contributed more than routine technical skills to solving the problem(s) addressed by the invention. (emphasis original)

See also Mr. Carver's pre-filing response (Garner Exhibit 12; partially expurgated):

As for the Inventors, we realise the importance of the choice with regards to patent legality and therefore suggest Ian Garner, Mike Dalrymple from PPL and Donna Prunkard and Don Foster from Zymo.

5. Using the standard "conception or reduction to practice" and considering Mr. Carver's views, I named Ian Garner, Michael A. Dalrymple, Donna E. Prunkard and Donald C. Foster, as co-inventors. Each, in my view, had made an intellectual contribution to either the conception or reduction to practice of a method to produce biocompetent fibrinogen in the milk of transgenic animals, and animals useful in that method.

I believe that I named Donna Prunkard and Donald Foster as inventors because they had decided to make fibrinogen in the milk of transgenic animals and made

the set of DNA constructs needed for the production. I believe that I named Ian Garner and Michael Dalrymple as inventors because I understood that they had provided the cloning vector to Donna Prunkard and had been involved in the production of the transgenic animals that produced biocompetent fibrinogen in their milk.

6. I now believe that the inventorship of the '940 patent and the application from which it issued is in error. Ian Garner and Michael A. Dalrymple should be deleted as co-inventors. After correction, co-inventors Donna E. Prunkard and Donald C. Foster will remain as the joint inventors of the '940 patent.


7. The error in my naming Ian Garner and Michael A. Dalrymple as co-inventors on the application that issued as the '940 patent arose without deceptive intent. As stated above, the error resulted from my considering as inventors those who made an intellectual contribution to either conception or reduction to practice of the claimed invention. At the time I made this determination, I erroneously did not separately consider conception and reduction to practice. I do not recall why I considered conception and reduction to practice together. I believe, however, that if I had followed the correct legal standard, I would have named only Donna E. Prunkard and Donald C. Foster as co-inventors.

8. I believe and understand that the error in inventorship was not discovered until I reviewed, in connection with counsel in *Garner v. Velandar*, Interference No. 104,242, the Garner application, the '940 patent in interference, and the work underlying the inventions claimed in the application and patent.

9. I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to

be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the above-identified patent.

Jan. 13, 1999  
Date

  
Gary E. Parker (Reg. No. 31,648)  
Principle Patent Agent  
ZymoGenetics, Inc.  
1201 Eastlake Avenue East  
Seattle, WA 98102

(3)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent No. : 5,639,940  
Patentee : Ian Garner, Michael A. Dalrymple\*, Donna E. Prunkard and Donald C. Foster  
Assignee : Pharmaceutical Proteins, Ltd., and ZymoGenetics, Inc.  
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For : PRODUCTION OF FIBRINOGEN IN TRANSGENIC ANIMALS

Hon. Assistant Commissioner for Patents  
Washington, D.C. 20231

CONSENT OF ASSIGNEE TO CORRECT INVENTORSHIP

Sir:

Pharmaceutical Proteins, Ltd., a co-assignee of the above-identified '940 patent, by virtue of an August 12, 1994 assignment of United States patent application 08/206,176, filed March 3, 1994, from Ian Garner and Michael A. Dalrymple, recorded at Reel 7166, Frame 0931, hereby consents to correct the inventorship of the '940 patent by deleting Ian Garner and Michael A. Dalrymple as named inventors and thus amending inventorship to Donna E. Prunkard and Donald C. Foster, jointly. 37 C.F.R. § 1.324(b)(3).

Pursuant to 37 C.F.R. § 3.73(b), the undersigned hereby states and certifies as follows:

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\* Dr. Dalrymple's middle initial was erroneously printed as "L" in the patent.

1. I am an officer of assignee corporation and am authorized to act on behalf of assignee corporation with respect to the above-identified '940 patent; and

2. The relevant evidentiary documents have been reviewed and, to the best of my knowledge and belief, an undivided share of the title to the '940 patent is in the assignee.

PHARMACEUTICAL PROTEINS, LTD.

15 Jan 1999  
Date

By: Alan Colman

Name: ALAN COLMAN

Title: RESEARCH DIRECTOR

(4)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent No. : 5,639,940  
Patentee : Ian Garner, Michael A. Dalrymple\*, Donna E. Prunkard and Donald C. Foster  
Assignee : Pharmaceutical Proteins, Ltd., and ZymoGenetics, Inc.  
Issued : June 17, 1997  
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Hon. Assistant Commissioner for Patents  
Washington, D.C. 20231

CONSENT OF ASSIGNEE TO CORRECT INVENTORSHIP

Sir:

ZymoGenetics, Inc., a co-assignee of the above-identified '940 patent, by virtue of an August 19, 1994 assignment of United States patent application 08/206,176, filed March 3, 1994, from Donna E. Prunkard and Donald C. Foster, recorded at Reel 7166, Frame 0921, hereby consents to correct the inventorship of the '940 patent by deleting Ian Garner and Michael A. Dalrymple as named inventors and thus amending inventorship to Donna E. Prunkard and Donald C. Foster, jointly. 37 C.F.R. § 1.324(b)(3).

Pursuant to 37 C.F.R. § 3.73(b), the undersigned hereby states and certifies as

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\* Dr. Dalrymple's middle initial was erroneously printed as "L" in the patent.

follows:

1. I am an officer of assignee corporation and am authorized to act on behalf of assignee corporation with respect to the above-identified '940 patent; and
2. The relevant evidentiary documents have been reviewed and, to the best of my knowledge and belief, an undivided share of the title to the '940 patent is in the assignee.

ZYMOGENETICS, INC.,

By: Shirko Campos

\_\_\_\_\_  
Date

Name: Shirko Campos

Title: Sr. V.P. Finance + Admin.



## Assembly and Secretion of Recombinant Human Fibrinogen\*

(Received for publication, July 23, 1990)

Samar N. Roy, Roman Procyk, Bohdan J. Kudryk, and Colvin M. Redman†

From the Lindsley F. Kimball Research Institute of The New York Blood Center, New York, New York 10021

Expression vectors containing full-length cDNAs for each of the human fibrinogen chains were constructed. COS-1 cells were transfected with single vectors, mixtures of two, or with all three vectors and stable cell lines selected. Cells transfected with single vectors, or with mixtures of any two vectors, expressed the appropriate fibrinogen chains but did not secrete them. COS cells transfected with three vectors expressed all of the chains and secreted fibrinogen. COS cells transfected with three vectors contained, intracellularly, a mixture of fibrinogen-related proteins. The four main intracellular products were nascent fibrinogen, an  $A\alpha$ - $\gamma$  complex, free  $A\alpha$  chains, and free  $\gamma$  chains. This is a similar pattern to that noted in Hep G2 cells. The intracellular forms of fibrinogen were sensitive to endoglycosidase H, indicating that they reside in a pre-Golgi compartment. Secreted fibrinogen was endoglycosidase H-insensitive, suggesting that the secreted glycoprotein moieties were processed in the normal manner. When mixed with plasma fibrinogen, radio-labeled recombinant fibrinogen was incorporated into a thrombin-induced clot. These studies demonstrate that COS cells transfected with all three fibrinogen chain cDNAs are capable of assembling and secreting a functional fibrinogen molecule.

Fibrinogen is composed of three different polypeptides ( $A\alpha$ ,  $B\beta$ , and  $\gamma$ ), arranged as a dimer with each half-molecule containing a set of each of the chains. The two half-molecules are linked together by three disulfide bonds at the  $NH_2$ -terminal portions of the polypeptides. Two of the symmetrical bonds are between adjacent  $\gamma$  chains and one is between  $A\alpha$  chains. In addition a complex set of inter- and intrachain disulfide bonds (there are 29 disulfide bonds with no free sulfhydryl groups) are involved in maintaining proper structure (1-4).

Our studies are aimed at determining how this multichain protein is synthesized, assembled, and secreted. Hepatocytes are the principal site of synthesis and each of the component chains of fibrinogen is encoded by a separate gene (5-8). Previously we demonstrated that Hep G2 cells have surplus pools of  $A\alpha$  and  $\gamma$  chains that occur either as free chains or complexed to each other, primarily as an  $A\alpha$ - $\gamma$  complex (9-11). Hep G2 cells maintain these surplus amounts of  $A\alpha$  and  $\gamma$  chains even when fibrinogen synthesis and secretion is stimulated by production of enhanced amounts of  $B\beta$  chain

(12). Pulse-chase experiments demonstrated that chain assembly commences by the attachment of preformed  $A\alpha$  and  $\gamma$  chains to nascent  $B\beta$  chains. On completion of  $B\beta$  chain elongation, the  $B\beta$ - $\gamma$  and  $B\beta$ - $A\alpha$  complexes are released into the lumen of the rough endoplasmic reticulum and acquire the third chain to form half-molecules. The two half-molecules are then joined to form dimeric fibrinogen. Chain assembly occurs in the rough endoplasmic reticulum (13).

To obtain further information on the mechanisms which govern chain assembly we prepared a set of stable transfected COS-1 cells which express either the individual fibrinogen chains, mixtures of two of the chains, or all three chains and studied the assembly and secretion of fibrinogen.

### EXPERIMENTAL PROCEDURES

#### Materials

L-[ $^{35}$ S]Methionine, approximately 1.1 Ci/mmol, was purchased from Du Pont-New England Nuclear, fetal calf serum from Hyclone, endoglycosidase H from Genzyme, geneticin from Sigma, restriction and modifying enzymes from Boehringer Mannheim, and T4 DNA ligase from New England Biolabs. Human fibrinogen (Imco, Stockholm), prepared as previously described (14), was stored at  $-70^\circ\text{C}$  as a stock solution of about 14 mg/ml in 50 mM Tris-HCl (pH 7.4) buffer containing 100 mM NaCl and 1 mM EDTA. The fibronectin present in this preparation was removed by affinity chromatography on gelatin-Sepharose (15). Fibrinogen concentration was measured spectrophotometrically in alkaline urea using  $E_{1\%}^{1\text{cm}} = 16.5$  at 282 nm. Human thrombin was obtained from the Department of Blood Coagulation Research, Karolinska Institutet, Stockholm, Sweden. Trasyol was from Bayer. Other reagents used have been described previously (9-12).

#### Cell Culture

COS-1 cells were maintained in Iacove's medium supplemented with 10% fetal calf serum (Hyclone) and 1% glutamine (16). Hep G2 cells were originally obtained in 1982 from Drs. Barbara B. Knowles and David P. Aden of the Wistar Institute, Philadelphia, PA; they were maintained in Eagle's minimal essential medium containing 10% fetal calf serum, 15 mM Tricine buffer, and penicillin/streptomycin (9, 10).

#### Construction of Expression Vectors

Full-length  $A\alpha$  and  $\gamma$  fibrinogen chain cDNAs were cloned into the *Pst*I site of BR322 (17, 18) and were kind gifts from Dr. Dominic Chung, University of Washington, Seattle, WA. Both  $A\alpha$  and  $\gamma$  cDNA have internal *Pst*I sites and both also have stop codons at the 5' end. Therefore, to obtain full length  $A\alpha$  and  $\gamma$  chain cDNAs, capable of being expressed, the following procedures were used to construct the expression vectors.

*pBC12BI-A $\alpha$* —The  $A\alpha$  cDNA was released from pBR322 by treatment with *Mst*I. The resulting 3.2-kb fragment (200 ng) was then digested with nuclease *Bal* 31 for 4 min to remove 50 bp from both ends so that the stop codon at position -28 together with 22 bp of pBR322 sequence was removed. The resulting DNA fragment, which

\* This study was supported by National Institutes of Health Grant HL-37457. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: COS, monkey kidney fibroblasts; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RSV, Rous sarcoma virus; kb, kilobase(s); Neo, neomycin-kanamycin-resistant gene; bp, base pair(s).

containing the full length coding region for  $\alpha$  fibrinogen chain, was purified and blunt ended with Klenow fragment followed by ligation with phosphorylated *Hind*III linker (10-mer, Boehringer Mannheim). This material was digested with *Hind*III/*Nco*I to create a *Hind*III site at the 5' end of the  $\alpha$  cDNA and a *Nco*I site at the 3' end.

To prepare pBC12BI to receive the above fragment, pBC12BI was digested with *Bam*HI/*Hind*III. The linear plasmid DNA was then ligated with phosphorylated *Bam*HI/Blunt adaptor (ds24/20-mer) from Boehringer Mannheim that contained a *Nco*I site at the other end. The plasmid DNA (100 ng) containing the adaptor was digested with *Nco*I and then ligated with  $\alpha$  cDNA (described above). The resulting circular expression vector, having the  $\alpha$  cDNA insert (Fig. 1A), was screened by digestion of a DNA miniprep with *Hum*HI/*Hind*III, which showed the presence of an insert of approximately 2 kb in the vector.

**pRSVNeo- $\alpha$ - $\alpha$  cDNA** was released from pBC12BI- $\alpha$  by digestion with *Bam*HI/*Hind*III. The released  $\alpha$  cDNA (200 ng) was ligated to 100 ng of pRSV Neo (19) that had been cut with *Hind*III and dephosphorylated with calf intestinal phosphatase. The linear vector containing  $\alpha$  cDNA, was filled-in with Klenow fragment and then self-ligated to form the circular expression vector (Fig. 1B). The correct orientation was determined by digesting the plasmid DNA with *Bam*HI/*Hind*III and selecting the clones that yielded fragments of 4.3 and 3.4 kb. The 4.3-kb fragment is composed of 2.0 kb of  $\alpha$  cDNA insert and 2.3 kb from the vector DNA.

**pBC12BI- $\gamma$** —The  $\gamma$  fibrinogen chain cDNA, which had been cloned in pBR322 (17) contains stop codons at position -39 and -42 of the 5' end. To obtain the full length coding region without these stop codons, the  $\gamma$  chain cDNA was released from pBR322 by digestion with *Sac*I and *Hind*III. The released  $\gamma$  chain cDNA (200 ng) was ligated to pBC12BI plasmid DNA (100 ng) that had been cut with *Hind*III, and then dephosphorylated. The linear vector containing  $\gamma$  chain cDNA was filled-in with Klenow fragment and then self-ligated to form the circular expression vector (Fig. 1C). The correct orientation was determined by digesting the plasmid DNA with *Hum*HI/*Hind*III and selecting the clones that yielded fragments of 5.3 kb and 390 bp. The 5.3-kb fragment is composed of 1.4 kb of  $\gamma$  cDNA insert and 3.9 kb from the vector DNA.

**pRSVNeo- $\gamma$** —The full-length  $\gamma$  cDNA (200 ng), prepared as described above, was ligated with 100 ng of pRSVNeo plasmid DNA that had been cut with *Hind*III and then dephosphorylated. As described above the linear vector containing  $\gamma$  cDNA was processed to form the circular expression vector (Fig. 1D). The correct orientation was determined by digesting the plasmid DNA with *Hum*HI/*Hind*III that yielded a 4.8-kb fragment containing 1.4 kb of  $\gamma$  cDNA and 3.4 kb of vector DNA, and a 2.25-kb fragment of vector DNA.

**pBC12BI-B $\beta$  and pRSVNeo-B $\beta$** —The expression vectors pBC12BI-B $\beta$  and pRSVNeo-B $\beta$ , containing full length B $\beta$  fibrinogen chain cDNA, were prepared as previously described (12, 16).

#### General Methods

All DNA fragments, obtained after restriction enzyme digestion, were purified by 1% agarose gel electrophoresis, electrophoretic transfer to a membrane, and hybridization.

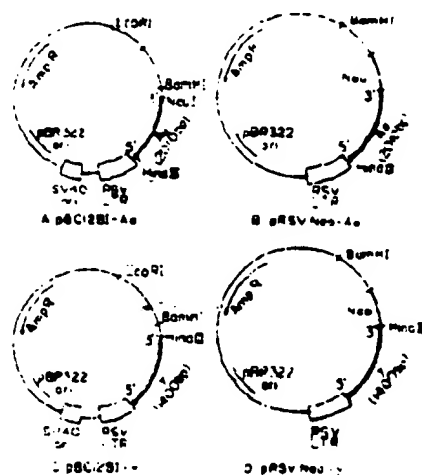


FIG. 1. Diagram of expression vectors, containing  $\alpha$  and  $\gamma$  fibrinogen chain cDNAs used to transfect COS-1 cells.

phenol-CHCl<sub>3</sub> extraction, and alcohol precipitation. Restriction enzyme digestions, dephosphorylation of vector DNA, fill in reactions with Klenow fragment, ligation of cDNAs with vector DNAs and phosphorylation of linker and adaptor were performed as described by Sambrook et al. (20).

Transformations with the constructed vectors were performed in *Escherichia coli* RRI competent cells on LB-agar plates containing 100  $\mu$ g/ml ampicillin. Miniprepations of plasmid DNA from selected bacterial colonies, were done by the alkaline-lysate method. To determine whether the cDNA inserts occurred in the correct orientation in the constructed vectors, the DNA was treated with appropriate restriction enzymes and the mobility of the DNA fragments determined by electrophoresis on 1% agarose gels. Large scale preparations of plasmid DNA were performed by alkaline lysis of bacterial preparations followed by cesium chloride-ethidium bromide equilibrium gradient centrifugation. All of the above general methods were performed by standard procedures (20).

#### Transfection and Selection of Stable Cell Lines

To determine whether the  $\alpha$  and  $\gamma$  cDNAs were expressed in COS-1 cells, the cells were first transiently transfected with either pBC12BI- $\alpha$  or pBC12BI- $\gamma$  by the calcium phosphate method (21) using 5  $\mu$ g of DNA/ml per  $2 \times 10^6$  cells in 60-mm culture dishes. The cells were metabolically labeled 48 h after transfection with L-[<sup>35</sup>S] methionine and the expressed fibrinogen chains determined by immunoprecipitation, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography, as previously described (9-11).

To obtain stable cell lines COS-1 cells were transfected by the calcium phosphate method (21) with either pRSVNeo-B $\beta$ , pRSVNeo- $\alpha$ , or pRSVNeo- $\gamma$  or with combinations of equal amounts of two of these expression vectors, or with equal amounts of all three expression vectors. In all cases 5  $\mu$ g of DNA/ml of each vector was used. When only one, or a mixture of two vectors were used pRSVNeo- $\alpha$  DNA was added to increase the DNA concentration to 15  $\mu$ g/ml. As a control, COS-1 cells were also transfected with pRSVNeo (15  $\mu$ g/ml) that did not contain fibrinogen cDNA inserts. The transfected cells were selected by resistance to 0.4 mg/ml geneticin for 5 weeks as previously described (12). After 2 weeks, two or three colonies remained in each of the transfected cell lines. After another 3 weeks the colonies were treated with trypsin, transferred to fresh plates, and allowed to grow to confluency in 60-mm plates in the presence of 0.4 mg/ml geneticin.

#### Incubation of Cells with L-[<sup>35</sup>S]Methionine

Before labeling with L-[<sup>35</sup>S]methionine the cells were kept for 24 h without geneticin in Jacow's medium supplemented with 10% fetal calf serum and 1% glutamine. The 90% confluent cells were then labeled for 2 h at 37°C in methionine-free Dulbecco's minimal essential medium (GIBCO) containing 200  $\mu$ Ci of L-[<sup>35</sup>S]methionine, 0.1 mg/ml heparin, and 1% glutamine (12).

#### Immunoprecipitation of Nascent Fibrinogen Chains

Radioactive fibrinogen chains were isolated by immunoprecipitation from cell lysates and from the cell medium. A rabbit polyclonal antibody that reacts with fibrinogen and its component chains was used (9, 10). Cell lysates were treated with indinostat, detergents, and proteolytic inhibitors prior to immunoprecipitation. The chains were separated by SDS-PAGE under reduced and nonreduced conditions and detected by autoradiography. The nonreduced polypeptides were excised from the gels, reduced with mercaptoethanol, and re-electrophoresed on SDS PAGE to identify the component chains. These procedures have previously been described (9-12).

Protein radioactivity was determined by cutting out the radioactive areas from the polyacrylamide gels and counting by liquid scintillation spectrometry (22). In some cases relative amounts of radioactivity were determined by scanning autoradiograms with a Shimadzu densitometer.

The following terminology is used to describe the stable transfected COS cells. COS- $\alpha$ , cells transfected with pRSVNeo- $\alpha$ ; COS- $\beta$ , transfected with pRSVNeo-B $\beta$ ; COS- $\gamma$ , transfected with pRSVNeo- $\gamma$ ; COS- $\alpha\beta$ , transfected with pRSVNeo- $\alpha$  and pRSVNeo-B $\beta$ ; COS- $\alpha\gamma$ , transfected with pRSVNeo- $\alpha$  and pRSVNeo- $\gamma$ ; COS- $\beta\gamma$ , transfected with pRSVNeo-B $\beta$  and pRSVNeo- $\gamma$ ; COS- $\alpha\beta\gamma$ , transfected with pRSVNeo- $\alpha$ , pRSVNeo-B $\beta$ , and pRSVNeo- $\gamma$ .

## Clotting of Recombinant Fibrinogen

The incubation medium of COS- $\alpha_2(\gamma)$  cells and of Hep G2 cells, incubated with L-[ $^{35}$ S]methionine for 2 h at 37 °C, was collected. An aliquot (0.75 ml) was treated with 220 units/ml final concentration of Trasylol, and mixed with purified human plasma fibrinogen (1.4 mg/ml) and  $\text{CaCl}_2$  (0.024 M). Some samples also contained 5 mM iodoacetamide to inhibit Factor XIII that is usually found in most plasma fibrinogen preparations. This treatment blocks cross-linking that occurs with fibrinogen and other proteins. Clotting was initiated by the addition of 3 units/ml thrombin. The clot was allowed to form overnight in a sealed Cones tube (Schleicher & Schuell) housing a 0.45- $\mu\text{m}$  cellulose acetate filter in which the bottom of the upper stage was sealed. The next day the clot was percolated with 0.05 M Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.4, until the radioactive background of the eluate had stabilized at its lowest level. The tube was then centrifuged to remove all liquid from the clot and the clot was hydrolyzed in 0.2 M NaOH containing 40% urea. The hydrolyzed DNA was neutralized with HCl and radioactivity determined. As a control, radioactive medium from COS cells transfected with an expression vector that did not contain fibrinogen chain cDNAs was treated in the same manner.

## Quantitation of Secreted Fibrinogen

The amount of secreted fibrinogen present in the medium of cells incubated for 24 h at 37 °C was determined by an indirect competition enzyme-linked immunosorbent assay procedure using a monoclonal antibody (Fd4-7B3) that is specific for an epitope in the  $\gamma$  chain of human fibrinogen fragment D (23). In brief, the assay procedure was as follows. Polyvinyl microtiter plates (Costar) were coated with pure human fibrinogen. An appropriate dilution of antibody was mixed with an equal volume of either buffer, pure human fibrinogen (concentration range of standard curve: 0.25–4.0  $\mu\text{g}/\text{ml}$ ), or media (from COS- $\alpha_2(\gamma)$  or Hep G2 cells). After mixing, each sample was added to the fibrinogen-coated enzyme-linked immunosorbent assay plate. Following incubation and subsequent wash cycles, an appropriate dilution of peroxidase-conjugated rabbit immunoglobulin to mouse immunoglobulin was added. Enzyme-linked IgG binding was detected using a  $\text{H}_2\text{O}_2$  and o-dianisidine solution.

## RESULTS

**Expression of Single and Combinations of Fibrinogen Chain cDNAs by COS Cells**—COS-1 cells were transfected with the expression vector pBC12BI containing either full length A $\alpha$  and  $\gamma$  chain cDNA and then, 48 h later, the cells were incubated with L-[ $^{35}$ S]methionine and the expression of radioactive fibrinogen chains determined. The cells expressed radioactive proteins which were immunoprecipitated with antibody to human fibrinogen. COS-cells transfected with pBC12BI-A $\alpha$  produced a radioactive protein which comigrated with authentic A $\alpha$  chains and COS cells transfected with pBC12BI- $\gamma$  expressed  $\gamma$  chains (data not shown). Cells transiently transfected with pBC12BI-B $\beta$  have previously been shown to express fibrinogen B $\beta$  chains (16).

Knowing that COS cells are capable of expressing A $\alpha$ , B $\beta$ , and  $\gamma$  chains of fibrinogen, we then transfected COS cells with pRSVNeo-A $\alpha$ , pRSVNeo-B $\beta$ , and pRSVNeo- $\gamma$  and selected stable transfected cell lines which were resistant to geneticin. Geneticin-resistant COS cells transfected with any one of the expression vectors expressed proteins which reacted with rabbit antibody to human fibrinogen and were of similar size to appropriate authentic human plasma fibrinogen chains. Cells transfected with combinations of two vectors containing different fibrinogen chain cDNAs (A $\alpha$  and B $\beta$ , A $\alpha$  and  $\gamma$ , and B $\beta$  and  $\gamma$ ) synthesized both chains and cells transfected with all three vectors expressed the three fibrinogen chains (Fig. 2).

Analyses, in nonreducing conditions, of the fibrinogen chains produced by cells transfected with combinations of two vectors showed that A $\alpha$  and B $\beta$ , A $\alpha$  and  $\gamma$ , and B $\beta$  and  $\gamma$  formed disulfide-linked complexes. The principal products and their molecular weights are A $\alpha$ -B $\beta$  complex ( $M_r$ , ~

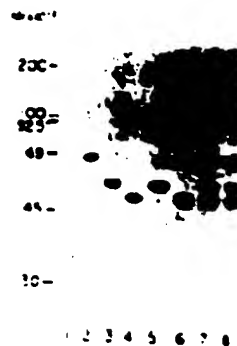


Fig. 2. Expression of fibrinogen chain cDNAs by COS cells. Stable transfected COS-1 cells were incubated for 2 h with L-[ $^{35}$ S]methionine and the fibrinogen chains synthesized were isolated by immunoprecipitation from cell lysates. The radioactive fibrinogen chains were separated by SDS-PAGE and detected by autoradiography. Autoradiograms are shown. Lane 1 is a control from cells transfected with pRSVNeo (without fibrinogen cDNA); lane 2, COS- $\alpha$  cells; lane 3, COS- $\beta$ ; lane 4, COS- $\gamma$ ; lane 5, COS- $\alpha,\beta$ ; lane 6, COS- $\alpha,\gamma$ ; lane 7, COS- $\beta,\gamma$ ; and lane 8, COS- $\alpha,\beta,\gamma$ . The location of standard molecular weight markers are shown on the left.



Fig. 3. Disulfide-linked complexes formed by expression of mixtures of fibrinogen chain cDNAs. The fibrinogen chains expressed by COS cells transfected with mixtures of two vectors containing different cDNAs were isolated from cell lysates as described in Fig. 2 and separated by SDS-PAGE. Autoradiograms are shown. In A, lane 1 contains the nonreduced immunoprecipitable protein expressed by COS- $\alpha,\beta$  cells, lane 2 contains that formed by COS- $\alpha,\gamma$  cells, and lane 3 contains that from COS- $\beta,\gamma$  cells. Molecular weight markers are given for each of the gels. In B, the samples were reduced with mercaptoethanol and the component chains re-electrophoresed. Lane 4 shows the fibrinogen chains expressed by COS- $\alpha,\beta,\gamma$  cells; lane 5, COS- $\alpha,\gamma$  cells; and lane 6, COS- $\beta,\gamma$  cells.

125,000), A $\alpha$ - $\gamma$  complex ( $M_r$ , 135,000), and B $\beta$ - $\gamma$  complex ( $M_r$ , 121,000) (Fig. 3). The size of the A $\alpha$ - $\gamma$  complex, as calculated from its electrophoretic mobility on SDS-PAGE is larger than predicted but not large enough to suggest the presence of a third chain. In addition to these principal products, small amounts of larger size complexes were also noted, but no free chains were detected (Fig. 3A). The chain composition of the complexes was determined by reduction with mercaptoethanol, and re-electrophoresis of the products. The complexes yielded a mixture of the expected two chains (Fig. 3B).

**Synthesis of Fibrinogen by COS- $\alpha_2(\gamma)$  Cells**—COS- $\alpha_2(\gamma)$  cells synthesized several fibrinogen-related proteins when analyzed under nonreducing conditions. The pattern noted is similar to that seen in Hep G2 cells (Fig. 4A). In COS- $\alpha_2(\gamma)$  cells, after 2 h of metabolic labeling with L-[ $^{35}$ S]methionine, 24.5% of the immunoprecipitable radioactivity was in fibrin-

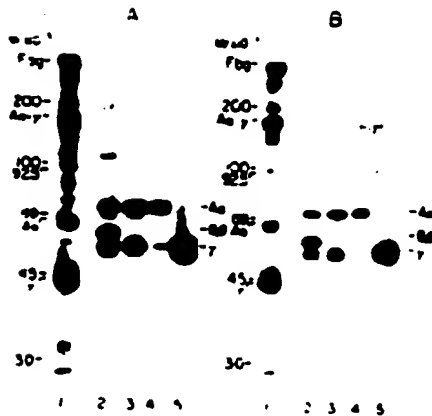


FIG. 4. Surplus A $\alpha$  and  $\gamma$  chains in Hep G2 cells and COS- $\alpha\beta\gamma$  cells. Hep G2 cells and COS- $\alpha\beta\gamma$  cells were incubated as in Fig. 2. The fibrinogen-related proteins in the cell lysates were separated by SDS-PAGE under nonreducing conditions. Lane 1, A is from Hep G2 cells and lane 1, B from COS- $\alpha\beta\gamma$  cells. Four of the major radioactive bands (marked Fbg, A $\alpha$ - $\gamma$ , A $\alpha$ , and  $\gamma$ ) were excised, reduced, and reexamined by SDS-PAGE. The chain compositions are shown in lanes 2-5. A is from Hep G2 cells and B from COS- $\alpha\beta\gamma$  cells. Lane 2, chain composition of reduced fibrinogen (Fbg); lane 3, reduced A $\alpha$ - $\gamma$ ; lane 4, reduced A $\alpha$ ; and lane 5, reduced  $\gamma$ . The location of standard molecular weight markers and of authentic A $\alpha$ , B $\beta$ , and  $\gamma$  chains from plasma fibrinogen are shown.

ogen, 22% in A $\alpha$ - $\gamma$  complex, 6% in free A $\alpha$  chains, and 41% in free  $\gamma$  chains. A parallel experiment with Hep G2 cells showed 31% in fibrinogen, 20% in A $\alpha$ - $\gamma$ , 9% in free A $\alpha$ , and 28% in free  $\gamma$  chains. Thus both stable transfected COS cells and Hep G2 cells develop surplus amounts of A $\alpha$  and  $\gamma$  chains which reside intracellularly mainly as free  $\gamma$  chain and as an A $\alpha$ - $\gamma$  complex.

The major intracellular forms of fibrinogen in COS- $\alpha\beta\gamma$  cells were characterized, as had been done previously for Hep G2 cells, by excision of the radioactive bands from the polyacrylamide gel, reduction, and re-electrophoresis (10). Their chain compositions and estimated molecular weights allowed us to identify these as fibrinogen, A $\alpha$ - $\gamma$  complex, and free A $\alpha$  and  $\gamma$  chains (Fig. 4B).

**Secretion**—COS cells which expressed single fibrinogen chains, and those which expressed two of the chains, in any combination, did not secrete these proteins into the medium (Fig. 5A). These single and duplex radioactive fibrinogen chains were only detected in the cell lysate (Figs. 2 and 3). However, COS- $\alpha\beta\gamma$  cells secreted the expressed proteins into the medium (Fig. 5A). When analyzed under nonreducing conditions the secreted fibrinogen chains were components of a high molecular weight disulfide-linked complex, with an apparent  $M_r$  of 340,000 which is similar to that of plasma fibrinogen. This  $M_r$  340,000 complex accounts for 99.4% of the immunoprecipitable protein radioactivity secreted. No free fibrinogen chains, nor intermediate products of assembly were detected in the medium (Fig. 5B). A small amount of protein radioactivity (less than 1%) was sometimes noted at about 130 kDa and this may be due to leakage from the cell or may be a degradative product of fibrinogen. A similar pattern was noted in the secretion of fibrinogen by Hep G2 cells (Fig. 5B). In the case of fibrinogen secreted by Hep G2 cells 90.4% of the immunoprecipitable radioactivity occurred as fibrinogen ( $M_r$  340,000) and a small amount, (~5%) was noted in a wide area, between 130 and 115 kDa (Fig. 5B).

Excision, reduction, and re-electrophoresis of the  $M_r$  340,000 radioactive protein secreted by COS- $\alpha\beta\gamma$  cells showed that this large protein was composed of A $\alpha$ , B $\beta$ , and

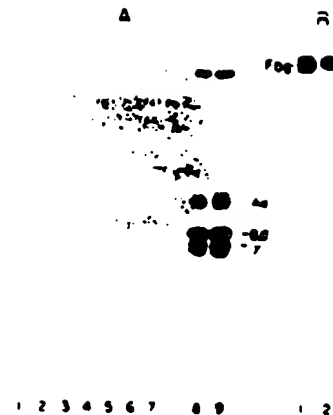


FIG. 5. Secretion of expressed fibrinogen chains. Transfected COS cells and Hep G2 cells were incubated for 2 h with L-[ $^{35}$ S] methionine and immunoprecipitable fibrinogen proteins secreted into the medium determined. In A, the immunoprecipitable proteins in the incubation medium were separated under reducing conditions and in B under nonreducing conditions. In A, lane 1, control COS cells transfected with pRSVNeo without fibrinogen cDNA; lane 2, COS- $\alpha$  cells; lane 3, COS- $\beta$  cells; lane 4, COS- $\gamma$  cells; lane 5, COS- $\alpha\beta$  cells; lane 6, COS- $\alpha\gamma$  cells; lane 7, COS- $\beta\gamma$  cells; lane 8, COS- $\alpha\beta\gamma$  cells. In B, lane 1, nonreduced fibrinogen secreted by Hep G2 cells; lane 2, nonreduced fibrinogen secreted by COS- $\alpha\beta\gamma$  cells.

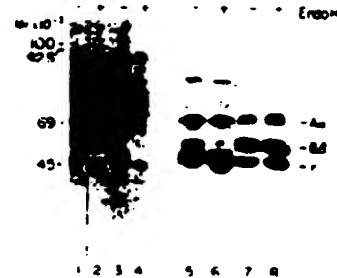


FIG. 6. Endoglycosidase H treatment of fibrinogen,  $\gamma$  chain, and A $\alpha$ - $\gamma$  chain complex. COS- $\gamma$ , COS- $\alpha\gamma$ , and COS- $\alpha\beta\gamma$  cells were incubated as in Fig. 2 and the secreted and nonsecreted fibrinogen chains were treated with endoglycosidase H. The proteins were reduced and separated by SDS-PAGE. Autoradiograms are shown. Proteins in lanes 1-4 were separated on different gels to those in lanes 5-8. Molecular weight markers are given on the left side for lanes 1-4 and on the right side for the location of authentic A $\alpha$ , B $\beta$ , and  $\gamma$  chains from plasma fibrinogen are given for lanes 5-8. Lanes 1-6 are nonsecreted immunoprecipitable fibrinogen proteins obtained from cell lysates and lanes 7 and 8 are secreted immunoprecipitable fibrinogen proteins. Lanes 1 and 2 are protein expressed by COS- $\alpha\gamma$  cells; lanes 3 and 4, COS- $\gamma$  cells; lanes 5 and 6, COS- $\alpha\beta\gamma$  cells. Lanes 7 and 8, fibrinogen secreted by COS- $\alpha\beta\gamma$  cells. -, not treated; +, treated with endoglycosidase H.

$\gamma$  chains. A similar pattern was noted in the fibrinogen secreted by Hep G2 cells (Fig. 5).

**Endoglycosidase H Treatment of Nonsecreted Chains**—A $\alpha$ - $\gamma$  complex and free  $\gamma$  chains expressed by transfected COS cells were treated with endoglycosidase H to determine whether nonsecreted  $\gamma$  chain contains mannose-rich oligosaccharides which are cleaved by the enzyme, or whether the carbohydrates of the  $\gamma$  chain had been further processed, making it insensitive to the enzyme (24-26). The  $\gamma$  chain of the A $\alpha$ - $\gamma$  complex on reduction, migrated on SDS-PAGE as a 48-kDa protein. On treatment with endoglycosidase H it migrated as a smaller 42-kDa protein (Fig. 6, lanes 1 and 2). The free  $\gamma$  chain, under nonreducing conditions, migrated as

a 48-kDa protein and on treatment with endoglycosidase H it migrated faster, as a 42-kDa protein (Fig. 6, lanes 3 and 4).

The B $\beta$  and  $\gamma$  chain components of intracellular fibrinogen, synthesized by COS- $\alpha$ , $\beta$ , $\gamma$  cells were also both endoglycosidase H-sensitive (Fig. 6, lanes 5 and 6). Thus, the major intracellular forms of fibrinogen, A $\alpha$ - $\gamma$  complex, the free  $\gamma$  chain, and nascent fibrinogen, are all endoglycosidase H-sensitive, indicating that they accumulate or are retained in a pre-trans-Golgi membrane compartment. Similar results were obtained when nascent intracellular fibrinogen, synthesized by Hep G2 cells, was analyzed (data not shown).

By contrast, the glycoprotein chains of secreted fibrinogen, produced by either COS- $\alpha$ , $\beta$ , $\gamma$  (Fig. 6, lanes 7 and 8) or Hep G2 cells (not shown), are endoglycosidase H-insensitive. This suggests that recombinant fibrinogen follows the conventional secretory pathway with normal glycosylation.

**Clotting of Recombinant Fibrinogen**—To determine whether secreted recombinant fibrinogen is capable of clotting, the incubation medium of control (nontransfected) and COS- $\alpha$ , $\beta$ , $\gamma$  cells, incubated for 24 h with L-[<sup>35</sup>S]methionine, was mixed with human plasma fibrinogen and induced to clot by the addition of thrombin. The clotting ability of recombinant fibrinogen was compared to that of fibrinogen secreted by Hep G2 cells. Clots formed in the presence of radiolabeled media from COS cells that were not transfected with the fibrinogen chain cDNAs had only background amounts of radioactivity associated with the clot matrix (2–3% of total trichloroacetic acid-precipitable radioactivity from the media). By contrast, clots formed in the presence of radiolabeled media from COS- $\alpha$ , $\beta$ , $\gamma$  cells, or from Hep G2 cells, had 30 to 45 times background levels of radioactivity associated with the clot. Clotting in the absence of Factor XIII cross-linking (i.e. in the presence of iodoacetamide) also produced highly radiolabeled clots, 18 to 24 times the background level. This indicates that the radiolabeled secreted fibrinogen became associated with the clot matrix through a thrombin-dependent polymerization mechanism.

**Amount of Recombinant Fibrinogen Secreted**—COS- $\alpha$ , $\beta$ , $\gamma$  cells secreted comparable amounts of fibrinogen as compared to Hep G2 cells. In two experiments COS- $\alpha$ , $\beta$ , $\gamma$  cells ( $2 \times 10^6$  cells) secreted an average of 2.08  $\mu$ g of fibrinogen in 24 h and the same number of Hep G2 cells secreted 1.94  $\mu$ g of fibrinogen.

## DISCUSSION

Fibrinogen is a multichain protein with a well ordered structure. It is sensitive to thrombin and acts in the final stages of blood clotting. Fibrinogen assembly which involves the arrangement of three different polypeptides into a symmetrical dimer probably occurs on structures within the endoplasmic reticulum which mediate proper alignment of the chains and also specific disulfide interactions. As such, a group of proteins known to be present in the lumen of the endoplasmic reticulum which probably include the immunoglobulin binding protein and protein disulfide isomerase are likely to be involved in a concerted effort to assemble the various chains into a functional molecule. (For reviews see Refs. 27, 28). Previously, the individual chains of fibrinogen have been expressed in surrogate cells, either *E. coli* (29–31) or in COS cells (16). However, expression, assembly, and secretion of fully formed, functional recombinant fibrinogen has not been reported. We show that COS cells, transfected with single fibrinogen chain cDNAs or with any combination of two fibrinogen chain cDNAs, express the appropriate fibrinogen chains but cannot secrete them. In contrast, COS cells containing all three fibrinogen chain cDNAs express, assemble,

and secrete the chains in a form which is capable of forming a thrombin-induced clot. This indicates that factors needed for proper assembly of fibrinogen chains are not restricted to the two tissues, hepatocytes and megakaryocytes, which normally express fibrinogen (32, 33). This further demonstrates that for fibrinogen chains to be properly transported and secreted they must exist as part of fully formed dimeric fibrinogen. This suggests that intact fibrinogen contains a signal which allows intracellular transport and secretion to occur and that individual chains are recognized as products not to be secreted.

*In vivo* free fibrinogen chains have not been detected in the circulation. In dogs, injected with radioactive amino acids, nearly all of the secreted fibrinogen chain radioactivity is accounted for in fibrinogen (34) and studies with cells in culture have indicated that fully formed fibrinogen is the main, if not the only form, of secreted fibrinogen chains (35, 36). This occurs in spite of the fact that in hepatocytes of several species studied, there is a surplus of two of the component chains of fibrinogen (9, 10, 35–37). In dogs (34) and rabbits (38) surplus chains have not been detected intracellularly, but different specific radioactivities of the component chains of secreted fibrinogen indicate that pools of A $\alpha$  and  $\gamma$  chains may occur. Thus, hepatocytes have a mechanism for distinguishing the surplus forms of fibrinogen chains from fully formed fibrinogen. A similar mechanism occurs in transfected COS cells. The stable transfected COS cell lines only secrete fully formed fibrinogen. Under nonreducing conditions 99.4% of the secreted immunoprecipitable protein is fibrinogen. There is less than 1% protein radioactivity in lower molecular weight proteins. As evidenced by sensitivity to endoglycosidase H treatment, the nonsecreted fibrinogen chains, and also nascent fibrinogen which is not yet fully processed, are retained in a pre-Golgi compartment. This is similar to the assembly and degradation of other heterologous proteins. In both the human asialoglycoprotein receptor (39) and the T-cell receptor proteins (40, 41) surplus chains are produced and some of these excess chains are degraded in a nonlysosomal pre-Golgi compartment.

Previous pulse-chase experiments, which carefully measured kinetic precursor-product relationships in Hep G2 cells, showed that surplus A $\alpha$  and  $\gamma$  fibrinogen chains participate in fibrinogen synthesis and assembly and that unused chains are retained and degraded intracellularly (9, 10). In COS- $\alpha$ , $\beta$ , $\gamma$  cells incubated for 2 h with L-[<sup>35</sup>S]methionine, which is near steady-state conditions, most of the radioactivity in fibrinogen chains occurs in three forms; as fully assembled fibrinogen whose carbohydrates have not been completely processed, as an A $\alpha$ - $\gamma$  complex and as free  $\gamma$  chains (Fig. 4). In addition, some free A $\alpha$  chains, and other intermediate forms, account for a small percentage of intracellular fibrinogen chains. This pattern is similar to that noted in Hep G2 cells and suggests that COS- $\alpha$ , $\beta$ , $\gamma$  cells assemble chains in a similar manner to Hep G2 cells. Kinetic pulse-chase experiments have not yet been performed with transfected COS cells and it is not clear whether all the intracellular precursor fibrinogen forms detected in Hep G2 cells are also present in transfected COS cells; or whether all of the intermediate forms present in COS cells participate in fibrinogen assembly. However, it is apparent that, as in hepatocytes of all species studied, surplus  $\gamma$  chains are generated in COS cells during fibrinogen assembly.

The mechanism by which surplus  $\gamma$  chains are generated is not understood. In Hep G2 cells, the initial rates of synthesis of the three chains are unequal with that of B $\beta$  being less than that of A $\alpha$  and  $\gamma$  (10). However, unequal degradative

rates have not been ruled out. In transfected COS cells the expression of the three fibrinogen chains is driven by the same viral promoter present in the expression vector pRSVNeo and thus regulation is unlikely to occur at the nuclear level; although we cannot rule out that different mRNAs are exported from the nucleus at different rates or that they have different stabilities. More likely the generation of surplus  $\gamma$  chains in transfected COS cells is a consequence of the chain assembly process and is probably a posttranslational event.

**Acknowledgment** We thank Gitali Mukhopadhyay for expert technical assistance.

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## Recombinant Human Fibrinogen and Sulfation of the $\gamma'$ Chain<sup>†</sup>

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Received May 21, 1991; Revised Manuscript Received July 24, 1991

**ABSTRACT:** Human fibrinogen and the homodimeric  $\gamma'$ -chain-containing variant have been expressed in BHK cells using cDNAs coding for the  $\alpha$ ,  $\beta$ , and  $\gamma$  (or  $\gamma'$ ) chains. The fibrinogens were secreted at levels greater than 4  $\mu$ g (mg of total cell protein)<sup>-1</sup> day<sup>-1</sup> and were biologically active in clotting assays. Recombinant fibrinogen containing the  $\gamma'$  chain incorporated <sup>35</sup>SO<sub>4</sub> into its chains during biosynthesis, while no incorporation occurred in the protein containing the  $\gamma$  chain. The identity of the sulfated  $\gamma'$  chain was verified by its ability to form dimers during clotting. In addition, carboxypeptidase Y digestion of the recombinant fibrinogen containing the  $\gamma'$  chain released 96% of the <sup>35</sup>S label from the sulfated chain, and the radioactive material was identified as tyrosine O-sulfate. These results clarify previous findings of the sulfation of tyrosine in human fibrinogen.

**H**uman fibrinogen is a soluble plasma protein that is converted to insoluble fibrin in the presence of thrombin. Fibrinogen (*M*<sub>r</sub> 340 000) is composed of two sets of three polypeptides, the  $\alpha$  (*M*<sub>r</sub> 66 000),  $\beta$  (*M*<sub>r</sub> 52 000), and  $\gamma$  (*M*<sub>r</sub> 46 500) chains (McKee et al., 1966). The six chains, ( $\alpha\beta\gamma$ )<sub>2</sub>, are extensively linked by disulfide bonds to form a complex trinodular structure (Hall & Slayter, 1959). During the coagulation cascade, the conversion of fibrinogen to a fibrin monomer occurs by the cleavage of amino-terminal fibrinopeptides from the  $\alpha$  and  $\beta$  chains (Bailey et al., 1951). This exposes polymerization sites which allow the fibrin monomers to interact and form the clot matrix (Laudano & Doolittle, 1978). The matrix is stabilized by the formation of  $\gamma$ -glutamyl- $\epsilon$ -lysine cross-links involving the  $\alpha$  and  $\gamma$  chains. This cross-linking reaction is catalyzed by factor XIIIa in the presence of calcium ions (Lorand et al., 1980; McKee et al., 1970) and results in a highly stable insoluble clot.

Fibrinogen is a multifunctional protein with many discrete domains. For example, fibrinogen (or fibrin) has binding sites for plasminogen (Lucas et al., 1983), tissue plasminogen activator (van Zonneveld et al., 1986), thrombin (Liu et al., 1979), and other plasma components. Fibrinogen also participates in platelet aggregation by binding to specific receptors on activated platelets (Marguerie et al., 1979; Hawiger et al., 1980). Accordingly, fibrinogen plays a central role in hemostasis and thrombosis.

The amino acid sequence of each of the three chains of human fibrinogen has been determined by amino acid sequence analysis (Blombäck et al., 1976; Doolittle et al., 1979; Henschen et al., 1980; Watt et al., 1979). In addition, the sequences of the cDNAs coding for the three chains (Chung et al., 1983a,b; Kant et al., 1983; Rixon et al., 1983) and their genes (Chung et al., 1990) have also been established. The three genes are clustered on chromosome 4 at position 4q23-32 (Henry et al., 1984) and occur in the order of  $\alpha$ ,  $\gamma$ , and  $\beta$ . The gene for the  $\beta$  chain is in the reverse orientation relative to the  $\alpha$  and  $\gamma$  genes (Kant et al., 1985). The  $\alpha$ ,  $\beta$ , and  $\gamma$  genes for human fibrinogen span approximately 45 kb and contain

four, seven, and nine introns, respectively (Chung et al., 1990; Kant et al., 1985).

A variant, nonallelic form of the  $\gamma$  chain which is found in about 10% of human plasma fibrinogen molecules (Francis et al., 1980; Wolfenstein-Todel & Mosesson, 1980) arises from the use of an alternative polyadenylation site within the ninth intron (Chung & Davie, 1984; Fornace et al., 1984) and is referred to as  $\gamma'$  (Wolfenstein-Todel & Mosesson, 1980),  $\gamma$ B (Francis et al., 1980), or  $\gamma^{57.5}$  (Peerschke et al., 1986). In the  $\gamma'$  chain, the carboxyl-terminal 4 amino acids have been replaced by a 20 amino acid segment (Wolfenstein-Todel & Mosesson, 1981). The function of the  $\gamma'$  chain is not known. Progress on its functional characterization has been hampered by the fact that  $\gamma'$ -containing fibrinogen has only been isolated as a heterodimer with the composition ( $\alpha\beta\gamma$ )( $\alpha\beta\gamma'$ ). Homodimeric ( $\alpha\beta\gamma'$ )<sub>2</sub> fibrinogen has not been isolated from plasma, thus providing a major impetus for the expression system presented here.

The three fibrinogen chains have been expressed individually in *Escherichia coli* (Lord, 1985; Bolyard & Lord, 1988, 1989), but functional fibrinogen has not been synthesized in a prokaryotic expression system. The first biologically active recombinant human fibrinogen was synthesized from cDNA clones in a mammalian cell expression system using baby hamster kidney (BHK)<sup>1</sup> cells (Farrell et al., 1989). Subsequently, a COS-1 cell expression system for the stable expression of fibrinogen from cDNA clones has also been developed (Roy et al., 1991); however, biological activity of the fibrinogen from this system was not shown. Another COS-1 expression system which produced biologically active, clottable fibrinogen has also been described by Hartwig and Danishefsky (1991) employing a transient expression system to identify potential intermediates in the assembly process.

Human fibrinogen has long been known to be sulfated on tyrosine residues (Jevons, 1963). Using the HepG2 hepatocellular carcinoma cell line (Knowles et al., 1980) which secretes human fibrinogen, Liu et al. (1985) identified the  $\beta$  chain of fibrinogen as the chain containing the sulfated tyrosine, based on the mobility of the  $\beta$  chain on SDS gels. The

<sup>†</sup> This work was supported by National Institutes of Health Grant HL 16919.

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<sup>1</sup> Abbreviations: BHK, baby hamster kidney; ELISA, enzyme-linked immunosorbent assay; rFbg, recombinant fibrinogen (containing  $\gamma$  chains); rFbg $\gamma'$ , recombinant fibrinogen (containing  $\gamma'$  chains).

site of sulfation, however, was different from that seen in bovine fibrinogen, which is sulfated on fibrinopeptide B (Bettelheim, 1954). Human fibrinogen lacks the corresponding tyrosine residue in its  $\beta$ -chain fibrinopeptide (Henschen et al., 1980; Watt et al., 1979). In addition, later studies in rat hepatocytes showed that rat fibrinogen was sulfated on tyrosine residues in the carboxyl end of the  $\gamma'$  chain (Hirose et al., 1988). In this paper, we present evidence that human fibrinogen is sulfated on the  $\gamma'$  chain, rather than the  $\beta$  chain as previously reported (Liu et al., 1985).

#### EXPERIMENTAL PROCEDURES

**Construction of Expression Vectors.** pAG-1 encoding the  $\alpha$  and  $\gamma$  cDNAs was constructed from the original cDNA clones in pBR322 (Rixon et al., 1983; Chung et al., 1983). The  $\alpha$  cDNA was subcloned as an *Asel*/*PvuI* fragment, filled in with T7 DNA polymerase, and cloned as a blunt-end fragment into the *HincII* site of M13mp18 to create pAM-1. A *Bam*HI site was constructed three nucleotides upstream from the initiator methionine with the oligonucleotide 5'-CATGCCTGCAGGTCGGATCCAAGATGTTTTCCA-TGAG-3' using an in vitro mutagenesis system (Amersham) to create pAM-2. In addition, a 793 base pair *Bgl*II/*Mlu*I fragment from the  $\alpha$  genomic clone pBS4 (Chung et al., 1990) was used to correct a mistake in the original  $\alpha$  cDNA in pH1 $\alpha$ 3 that contained a deletion of the codon TCT for Ser-417. The  $\gamma$  cDNA was subcloned by cleavage at the 5' *Sst*I site and the 3' *Hind*III site and inserted into the *Sst*I and *Hind*III sites in pIC19R (Marsh et al., 1984) to create pGI-1. A *Bgl*II/*Bam*HI fragment was subcloned from pGI-1 into the *Bam*HI site of Zem86 (Mulvihill et al., 1988) to create pGI-2. This placed the  $\gamma$  cDNA under the control of the SV-40 early promoter/enhancer element (Subramani et al., 1981). The human growth hormone transcriptional terminator was added at the 3' end, since the *Hind*III cleavage removed the endogenous  $\gamma$  terminator. The terminator was identical with that from MTHGH111 (Palmiter et al., 1983). The entire  $\gamma$  transcription unit including promoter and terminator was subcloned as an *Eco*RI fragment into pMT-1, creating pMG-1. This placed a metallothionein promoter (Palmiter et al., 1983) in the opposite transcriptional orientation to the SV-40 promoter. The metallothionein promoter was originally derived from MTHGH111, as cloned in Zem93 (Mulvihill et al., 1988). The final step in the construction was the insertion of the modified  $\alpha$  cDNA as a *Bam*HI fragment from pAM-2 into the *Bam*HI site in pMG-1 to create pAG-1. Plasmid pAG- $\gamma'$ , which encodes the  $\alpha$  and  $\gamma'$  chains, was constructed in the same sequence shown for pAG-1, except that the 3' end of the  $\gamma$  DNA in pGI-2 was replaced with the 758 bp *Pst*I fragment encoding the  $\gamma'$ -carboxyl terminus. In both pAG-1 and pAG- $\gamma'$ , the  $\alpha$  cDNA is transcribed from the metallothionein promoter, and the  $\gamma$  (or  $\gamma'$ ) cDNA is transcribed in the opposite direction from the SV-40 promoter.

The  $\beta$  cDNA was cloned into the *Pst*I site of M13mp18 to create pBM-1. A new *Pst*I site was constructed three nucleotides upstream from the initiator methionine with the oligonucleotide 5'-CTTGCATGCCTGCAGACCATGA-AACATCTATTA-3' to create pBM-2. The SV-40 promoter/enhancer element and the dihydrofolate reductase cDNA originally present in pSV2 DHFR (Subramani et al., 1981) were subcloned as an *Eco*RI fragment from Zem176 (Mulvihill et al., 1988) into Zem93 to create pMD-1, which has a metallothionein promoter in the opposite transcriptional orientation to the SV-40 promoter. The modified  $\beta$  cDNA from pBM-2 was subcloned as a *Pst*I fragment into the *Pst*I site in pMD-1 to create pBD-1. Transcription of the  $\beta$  cDNA

from the metallothionein promoter was in the opposite direction from transcription of the dihydrofolate reductase cDNA from the SV-40 promoter.

The construction of expression vectors pAG-1, pAG- $\gamma'$ , and pBD-1 was carried out by established techniques (Sambrook et al., 1989). The sequences of the modified cDNAs were confirmed by using a dideoxy chain termination system (United States Biochemical Corp).

**Cell Culture.** HepG2 human liver cells (Knowles et al., 1980) were grown in minimum essential medium/10% fetal bovine serum/1 mM sodium pyruvate/0.1 mM nonessential amino acids/100  $\mu$ g/mL neomycin/50  $\mu$ g/mL penicillin/50  $\mu$ g/mL streptomycin (Gibco) in a 5% CO<sub>2</sub> atmosphere at 37 °C. A thymidine kinase deficient baby hamster kidney cell line, BHK 570 (ATCC CRL 10314), containing the expression vector pPAB-5 (Busby et al., 1991) for  $\alpha_2$ -antiplasmin (a generous gift from Dr. Don Foster; ZymoGenetics, Inc., Seattle, WA) was used as the host cell for the transfections and was grown under the same conditions in Dulbecco's modified Eagle medium/5% fetal bovine serum/100  $\mu$ g/mL neomycin/50  $\mu$ g/mL penicillin/50  $\mu$ g/mL streptomycin (Gibco). For transfections, BHK cells were plated at 1:25 split ratios in 150-mm plates (Falcon) overnight and transfected with 25  $\mu$ g of calcium phosphate precipitated pAG-1 (or pAG- $\gamma'$ ) and pBD-1 (12.5  $\mu$ g of each) for 4 h in 10 mL of medium. After a 1-min shock in 15% glycerol/Tris-buffered saline, cells were grown for 24 h in normal medium. The cells were then grown in selective medium with 20  $\mu$ M methotrexate for 7–10 days and screened by using an immunofilter assay (McCracken & Brown, 1984). The highest producing clones isolated were designated BHK-Fbg and BHK-Fbg $\gamma'$ , which secreted fibrinogen (rFbg) and  $\gamma'$ -containing homodimeric fibrinogen (rFbg $\gamma'$ ), respectively.

**Immunoprecipitation and Clotting Assays.** Confluent BHK-570, BHK-Fbg, HepG2, and BHK-Fbg $\gamma'$  cells in 24-well plates (Corning) were washed twice with 2 mL of 120 mM NaCl/2.7 mM KCl/10 mM sodium phosphate, pH 7.4 (phosphate-buffered saline), and metabolically labeled for 24 h in 0.5 mL of Dulbecco's modified Eagle medium containing 20 mM Hepes (pH 7.4)/3.7 g/L sodium bicarbonate/100  $\mu$ g/mL neomycin/50  $\mu$ g/mL penicillin/50  $\mu$ g/mL streptomycin. For [<sup>35</sup>S]cysteine labeling, 100  $\mu$ Ci/mL [<sup>35</sup>S]cysteine (>600 Ci/mmol, Amersham) was added to cysteine-free medium (JRH Biosciences). For <sup>35</sup>SO<sub>4</sub> labeling, 100  $\mu$ Ci/mL <sup>35</sup>SO<sub>4</sub> (25–40 Ci/mg, Amersham) was added to sulfate-free medium (JRH Biosciences). Control cells without label were also used in ELISA assays to be described below in order to quantitate expression levels. Total cell protein in the cell monolayers was determined by using the BCA assay (Pierce) on cells extracted in RIPA buffer (Sambrook, 1989), using bovine serum albumin as a standard.

For immunoprecipitations, the medium described above containing 50  $\mu$ g/mL benzamidine (Sigma)/1  $\mu$ M leupeptin (Boehringer Mannheim)/50  $\mu$ g/mL soybean trypsin inhibitor (Sigma) was used for labeling. After the 24-h incubation, the medium was removed, and protease inhibitors were added at the following concentrations: 5 mM 6-amino-*n*-hexanoic acid (Sigma)/5 mM EDTA (Sigma)/0.1 mM *n*-ethylmaleimide (Sigma)/1  $\mu$ M pepstatin A (Boehringer Mannheim)/0.2 mM phenylmethanesulfonyl fluoride (Sigma). The cells were washed twice with 2 mL of phosphate-buffered saline and solubilized in 0.5 mL of RIPA buffer containing the above protease inhibitors. All subsequent incubations were done at 4 °C with rocking. The labeled material was preadsorbed with 5  $\mu$ L of normal rabbit serum for 1 h and precipitated with 2



mg of protein A-Sepharose (Sigma) for 1 h. The protein A-Sepharose was pelleted by centrifugation in a microfuge for 30 s at 4 °C. A total of 2.5  $\mu$ L of a rabbit anti-human fibrinogen antiserum (Behring) was added to the remaining supernatant and incubated for 1 h. Two milligrams of protein A-Sepharose was added for 1 h and washed with 1 mL of RIPA, 1 mL of 0.5 M NaCl/20 mM Tris (pH 7.4)/1% NP-40/1 mM EDTA, and 1 mL of 0.15 M NaCl/20 mM Tris (pH 7.4)/1 mM EDTA (all wash buffers contained the above protease inhibitors). Sample buffer (100  $\mu$ L) with or without 5% 2-mercaptoethanol was added to the pellet and boiled 5 min before being loaded on gels (Laemmli, 1970).

For clotting assays, cells were labeled in the absence of protease inhibitors. After 24 h, the medium was removed, and 25  $\mu$ L of human plasma (George King Biomedical) was added and allowed to clot for 3 h at room temperature. The clots were centrifuged 10 min at 4 °C in a microfuge, and the pellets were washed and solubilized with the same buffers used for the immunoprecipitations.

<sup>14</sup>C-Labeled molecular weight markers were obtained from Bethesda Research Laboratories. The samples were run on 10% gels according to Laemmli (1970), impregnated with Amplify (Amersham) according to the manufacturer's directions, dried, and exposed to XAR-5 film (Kodak) with intensifying screens (Cronex) at -70 °C.

**Assay for Fibrinogen.** An ELISA for fibrinogen was developed by using the procedure of Flaherty et al. (1990). Briefly, an affinity-purified IgG fraction from a rabbit polyclonal antiserum to human fibrinogen (Accurate Chemical & Scientific Corp.) was biotinylated using biotin-amidocaproate *n*-hydroxysuccinimide ester (Sigma) and used for the detection of antibody-bound immobilized fibrinogen in ELISA plate wells (Corning). Streptavidin-alkaline phosphatase (BRL) was used to detect the biotinylated antibody. The phosphatase substrate used was *p*-nitrophenyl phosphate (Sigma). The limit of detection of the assay was below 1 ng/mL fibrinogen. Normal plasma fibrinogen isolated by glycine precipitation (Kazal et al., 1963) was used as the standard.

**Tyrosine O-Sulfate Analysis.** <sup>35</sup>S-Labeled rFbg $\gamma'$  was isolated by immunoprecipitation of <sup>35</sup>SO<sub>4</sub>-labeled BHK-Fbg $\gamma'$  cells as described above, except that 100-mm plates of cells were labeled with 5 mL of medium containing 200  $\mu$ Ci/mL <sup>35</sup>SO<sub>4</sub>. The immunoprecipitate from 1 mL of medium, which included the protein A-Sepharose, antibody, and labeled rFbg $\gamma'$ , was digested for 24 h at 37 °C in 100  $\mu$ L of 50 mM sodium acetate, pH 5.5, containing 0.5  $\mu$ g/mL carboxypeptidase Y (Calbiochem; 143.6 units/mg). The reaction mixture was centrifuged in a microfuge for 30 s, and 10  $\mu$ L of the supernatant was precipitated with 90  $\mu$ L of acetone for 30 min at 4 °C. The precipitate was removed by centrifugation for 10 min in a microfuge, and the supernatant was evaporated until it was dry.

For amino acid analysis, the dried acetone supernatant (approximately 2600 cpm) was derivatized with phenyl isothiocyanate and chromatographed on a WISP C18 system (Waters) as previously described (Bidlingmeyer, 1984). Fractions (0.5 mL) were collected every 0.5 min and mixed with 5 mL of Ecolume (ICN) for scintillation counting. Tyr O-sulfate standard (200 pmol), kindly provided by Dr. Ming-Cheh Liu (University of Oklahoma, Norman, OK), was derivatized and chromatographed in the same manner, and the effluent was monitored at 254 nm.

## RESULTS

**Secretion of Recombinant Fibrinogens from BHK Cells.** The baby hamster kidney cell line BHK 570, which is deficient

in thymidine kinase, was chosen as the host cell line for transfection because of its ability to express many proteins of the coagulation and fibrinolytic pathways, and its ability to allow amplification of expression vectors containing the dihydrofolate reductase selectable marker. A derivative of this cell line which expresses  $\alpha_2$ -antiplasmin was used in order to minimize proteolysis of the expressed fibrinogen secreted into the medium. Cotransfection of these BHK cells with pAG-1 and pBD-1 (Figures 1 and 2) and selection in 20  $\mu$ M methotrexate resulted in several colonies secreting rFbg. The parental BHK cell line did not produce detectable fibrinogen, using an assay capable of detecting less than 1 ng/mL. A stable cell line (BHK-Fbg) produced rFbg at levels of 1.1  $\mu$ g mL<sup>-1</sup> day<sup>-1</sup> at confluence in 24-well plates. To produce homodimeric  $\gamma'$ -containing fibrinogen (rFbg $\gamma'$ ), BHK cells were transfected with pAG- $\gamma'$  and pBD-1, resulting in cell line BHK-Fbg $\gamma'$ . Similar secretion levels of 0.83  $\mu$ g mL<sup>-1</sup> day<sup>-1</sup> were achieved with BHK-Fbg $\gamma'$ , which produced rFbg $\gamma'$ . Each recombinant cell line produced slightly less fibrinogen at confluence than the HepG2 cell line (1.3  $\mu$ g mL<sup>-1</sup> day<sup>-1</sup>). When the data were normalized to the total amount of cellular protein in the wells, however, the recombinant cell lines produced more fibrinogen per unit of total cellular protein. HepG2 cells produced 3.0  $\mu$ g (mg of protein)<sup>-1</sup> day<sup>-1</sup>, while BHK-Fbg and BHK-Fbg $\gamma'$  produced 5.0 and 4.0  $\mu$ g (mg of protein)<sup>-1</sup> day<sup>-1</sup>, respectively.

rFbg comigrated with normal human fibrinogen from HepG2 cells on unreduced SDS-polyacrylamide gel electrophoresis (Figure 3, lanes 2 and 3). Upon reduction, the recombinant  $\alpha$ ,  $\beta$ , and  $\gamma$  chains also comigrated with their normal counterparts (lanes 6 and 7). Similarly, rFbg $\gamma'$  migrated as a high molecular weight complex under nonreducing conditions (lane 4). Upon reduction, the  $\alpha$  and  $\beta$  chains comigrated with the HepG2 chains, while the  $\gamma'$  chain migrated at a slightly higher position than the  $\gamma$  chain, consistent with the greater molecular weight of the  $\gamma'$  chain (lanes 7 and 8). These results indicate that the recombinant fibrinogens had the correct ( $\alpha\beta\gamma$ )<sub>2</sub> or ( $\alpha\beta\gamma'$ )<sub>2</sub> composition.

**Biological Activity of the Recombinant Fibrinogens.** The recombinant fibrinogens were assayed for functional activities which are essential physiological features of normal fibrinogen. These include the ability of fibrinogen to be incorporated into a fibrin clot and the ability of fibrin monomers to be cross-linked by factor XIIIa. Incorporation into a fibrin clot and subsequent cross-linking require at minimum (1) cleavage of fibrinopeptide A by thrombin, exposing polymerization sites in the resulting fibrin monomers, and (2) proper alignment in the fibrin matrix, such that the  $\alpha$  and  $\gamma$  (or  $\gamma'$ ) chains in adjacent fibrin monomers are correctly oriented for cross-linking by factor XIIIa.

In order to test the clottability of the recombinant fibrinogens, it was necessary to use an assay which could detect relatively low amounts of recombinant fibrinogens produced by the BHK cells. Standard clotting assays which rely on 2–4 mg/mL fibrinogen were unsuitable. Metabolically labeled recombinant fibrinogens were therefore used for the clotting assays. Figure 3 shows that recombinant fibrinogens labeled with [<sup>35</sup>S]cysteine were incorporated into a fibrin clot and were readily cross-linked. In lanes 9–12, the labeled medium was clotted with normal human plasma prior to electrophoresis. The washed, solubilized clots from BHK, BHK-Fbg, HepG2, and BHK-Fbg $\gamma'$  media are shown in lanes 9, 10, 11, and 12, respectively. In both BHK-Fbg and HepG2 fibrin clots, the  $\alpha$ - and  $\gamma$ -chain bands diminished in intensity, consistent with their conversion by factor XIIIa to multimers and cross-linked

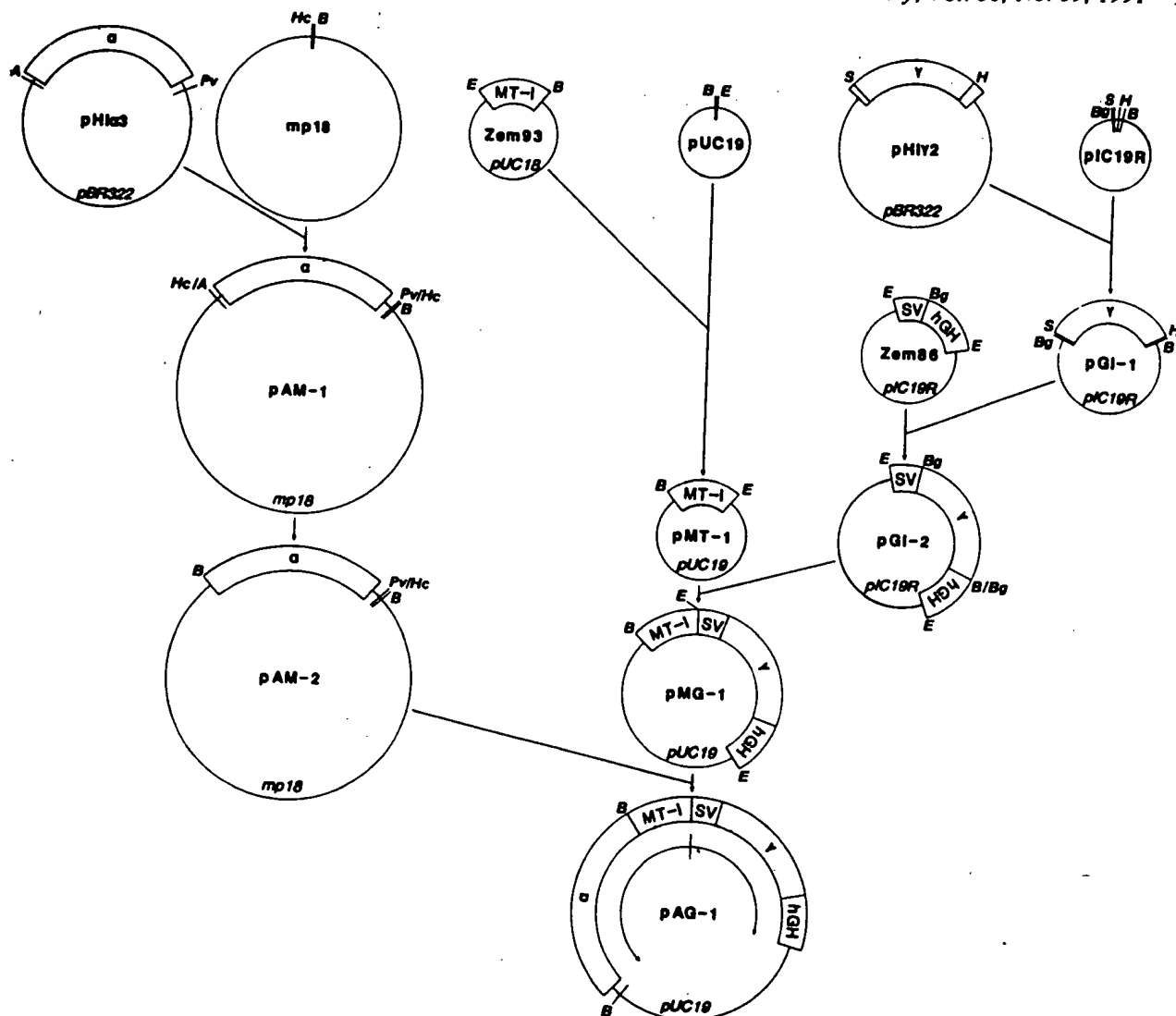


FIGURE 1: Construction of pAG-1. The 5'-untranslated regions of the original  $\alpha$  and  $\gamma$  cDNAs in pHI $\alpha$ 3 and pHI $\gamma$ 2 were removed after being cloned into M13mp18 and pIC19R, respectively, to create pAM-2 and pGI-1. The  $\alpha$  cDNA was placed under the control of a modified metallothionein promoter, while the  $\gamma$  cDNA was placed under the control of the SV-40 early promoter/enhancer. The direction of transcription is shown by the curved arrows. A = *Ase*I, B = *Bam*HI, Bg = *Bgl*II, E = *Eco*RI, H = *Hind*III, Hc = *Hinc*II, Pv = *Pvu*I, S = *Sst*I.

dimers, respectively (lanes 10 and 11). The  $\beta$ -chain bands shifted to a lower position on the gel, probably due to proteolytic degradation, as seen by other investigators (Lucas et al., 1983). Concomitant with the disappearance of the  $\gamma$  bands, new bands appeared at the position of cross-linked  $\gamma$ - $\gamma$  dimers.

Similarly, rFbg $\gamma'$  was also incorporated into a fibrin clot. Lane 12 shows that after clotting, the  $\beta$  band comigrated with those from both BHK-Fbg and HepG2 fibrinogen. The  $\alpha$  band and  $\gamma'$  band decreased in intensity, while a new band appeared at a position slightly above the  $\gamma$ - $\gamma$  dimer. Since the unlabeled human plasma used to form the clot contributed much more fibrinogen in this assay than the BHK-Fbg $\gamma'$  cells ( $\sim 75 \mu\text{g}$  vs  $\sim 0.5 \mu\text{g}$ ), this new band is most likely a  $\gamma'$ - $\gamma$  heterodimer. These results indicate that both the recombinant and HepG2 fibrinogens were incorporated into fibrin clots in the correct orientation, such that the  $\alpha$  and  $\gamma$  (or  $\gamma'$ ) chains acted as substrates for factor XIIIa. Furthermore, in preliminary experiments, it was shown that rFbg bound to platelets in a dose-dependent manner which paralleled the binding of plasma fibrinogen (unpublished results). Therefore, the recombinant fibrinogens appear to be biologically active using several important criteria, including incorporation into fibrin clots and cross-linking by factor XIIIa.

**Sulfation of Fibrinogen.** The recombinant fibrinogens were examined for sulfation in order to determine if any differences in this posttranslational modification existed between rFbg and rFbg $\gamma'$ . BHK-Fbg and BHK-Fbg $\gamma'$  cells were labeled with  $^{35}\text{SO}_4$  to visualize the sulfated chains, and parallel cell cultures were labeled with [ $^{35}\text{S}$ ]Cys to unambiguously identify the three chains of fibrinogen. The labeled medium was then either immunoprecipitated or clotted with normal human plasma. Figure 4 shows that only the  $\gamma'$  chain incorporated detectable amounts of  $^{35}\text{SO}_4$ . Lanes 1 and 2 show rFbg labeled with [ $^{35}\text{S}$ ]Cys and  $^{35}\text{SO}_4$ , respectively, and immunoprecipitated. No  $^{35}\text{SO}_4$ -labeled bands are seen in lane 2. In contrast, rFbg $\gamma'$  labeled with [ $^{35}\text{S}$ ]Cys and  $^{35}\text{SO}_4$  (lanes 3 and 4, respectively) showed labeling of a band with  $^{35}\text{SO}_4$  which comigrated with the  $\gamma'$  band. These data show that only the  $\gamma'$  chain was sulfated to an appreciable extent.

To ensure that the  $^{35}\text{SO}_4$ -labeled band was indeed the  $\gamma'$  band and not a proteolyzed  $\beta$  or  $\alpha$  band, the labeled media were clotted, and the solubilized clot was run on a gel. As shown earlier in Figure 3, the clotted fibrinogen had a characteristic binding pattern in which the  $\alpha$  bands decreased sharply in intensity, the  $\beta$  band shifted to a slightly lower position, and the  $\gamma$  (or  $\gamma'$ ) band shifted to the dimer position. Figure 4, lanes 5 and 6, shows [ $^{35}\text{S}$ ]Cys- and  $^{35}\text{SO}_4$ -labeled

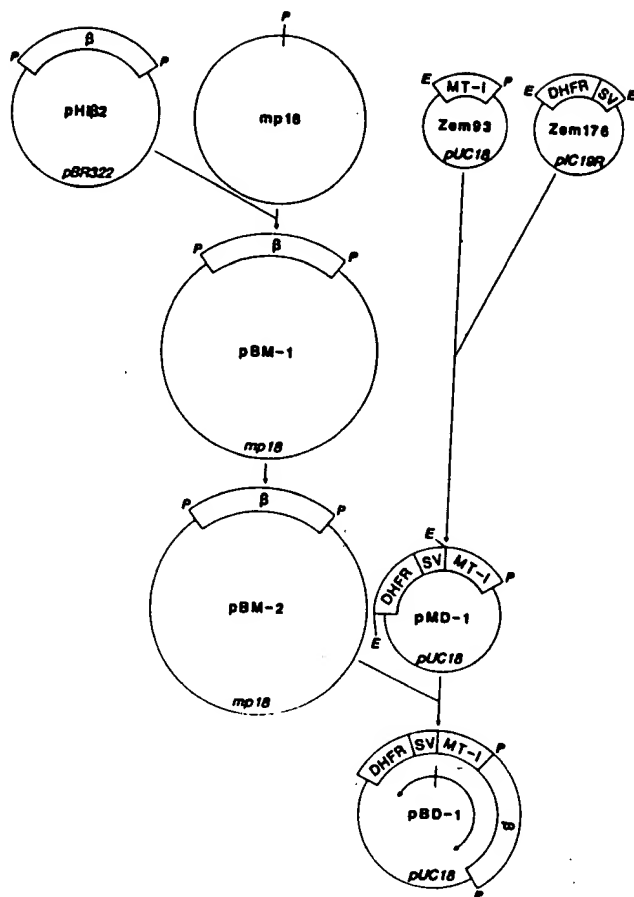


FIGURE 2: Construction of pBD-1. The 5'-untranslated region of the  $\beta$  cDNA in pHI82 was removed after being cloned into M13mp18 to create pBM-2. The  $\beta$  cDNA was placed under the control of a modified metallothionein promoter, while the dihydrofolate reductase selectable marker cDNA was under the control of the SV-40 early promoter/enhancer. The direction of transcription is shown by the curved arrows. E = EcoRI, P = PstI.

rFbg, respectively, after clotting. The  $\beta$  band and the  $\gamma$ - $\gamma$  dimer are apparent in the [ $^{35}$ S]Cys-labeled rFbg (lane 5), but not in the [ $^{35}$ SO $_4$ ]-labeled rFbg (lane 6). However, rFbg $\gamma'$  shows the  $\gamma$ - $\gamma'$  dimer band after labeling with both [ $^{35}$ S]Cys (lane 7) and [ $^{35}$ SO $_4$ ] (lane 8), indicating that the  $\gamma'$  chain was readily sulfated. Significantly, no [ $^{35}$ SO $_4$ ]-labeled band was detected at the position of the  $\beta$  or  $\alpha$  bands. These results confirm the sulfation of the  $\gamma'$  chain.

**Tyrosine O-Sulfate Analysis.** The [ $^{35}$ SO $_4$ ]-labeled rFbg $\gamma'$  was analyzed to determine whether the label was incorporated into Tyr residues. Carboxypeptidase Y treatment of the immunoprecipitated rFbg $\gamma'$  caused the release of 45% of the label after 30 min and 96% of the label after a 24-h digestion. The released labeled material was derivatized with phenyl isothiocyanate and chromatographed on a C18 column for amino acid analysis. A major peak of radioactivity which corresponded to 81% of the input [ $^{35}$ S] label eluted 11.5 min after injection (Figure 5, upper panel), in addition to a minor breakthrough peak at 3.5 min and an unidentified minor peak at 15.5 min. In comparison, a derivatized Tyr O-sulfate standard eluted at 11.8 min after injection (Figure 5, lower panel), with a byproduct peak at 19.8 min. The elution times of the major [ $^{35}$ S] peak and the Tyr O-sulfate standard indicate that the majority of the sulfation in the  $\gamma'$  chain occurs on Tyr.

#### DISCUSSION

Fibrinogen is the central molecule in the blood coagulation cascade and forms the structural basis of the fibrin clot. As such, it is essential for normal hemostasis in vivo. Although

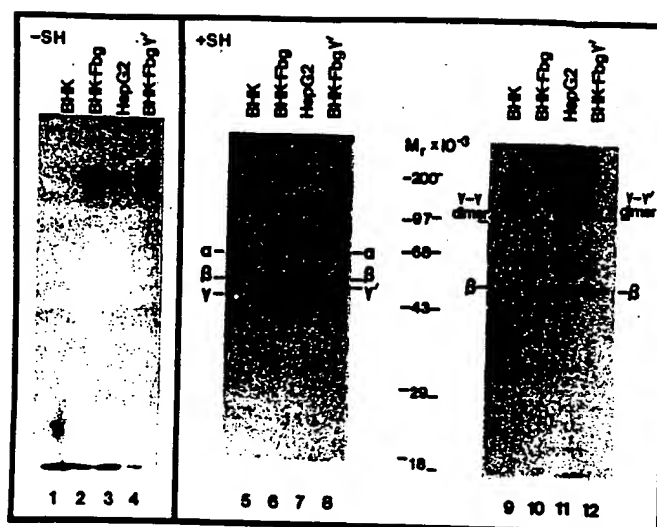


FIGURE 3: [ $^{35}$ S]-labeled fibrinogens. [ $^{35}$ S]Cysteine-labeled fibrinogens from BHK, BHK-Fbg, HepG2, and BHK-Fbg $\gamma'$  cells were immunoprecipitated or clotted and run on gels. Lanes 1-4 show normal and recombinant fibrinogens immunoprecipitated and run on 5% nonreduced gels: (1) BHK cells; (2) BHK-Fbg cells; (3) HepG2 cells; (4) BHK-Fbg $\gamma'$  cells. Lanes 5-8 show normal and recombinant fibrinogens immunoprecipitated and run on 10% reduced gels: (5) BHK cells; (6) BHK-Fbg cells; (7) HepG2 cells; (8) BHK-Fbg $\gamma'$  cells. Lanes 9-12 show normal and recombinant fibrinogens clotted with human plasma and run on 10% reduced gels: (9) BHK cells; (10) BHK-Fbg cells; (11) HepG2 cells; (12) BHK-Fbg $\gamma'$  cells.

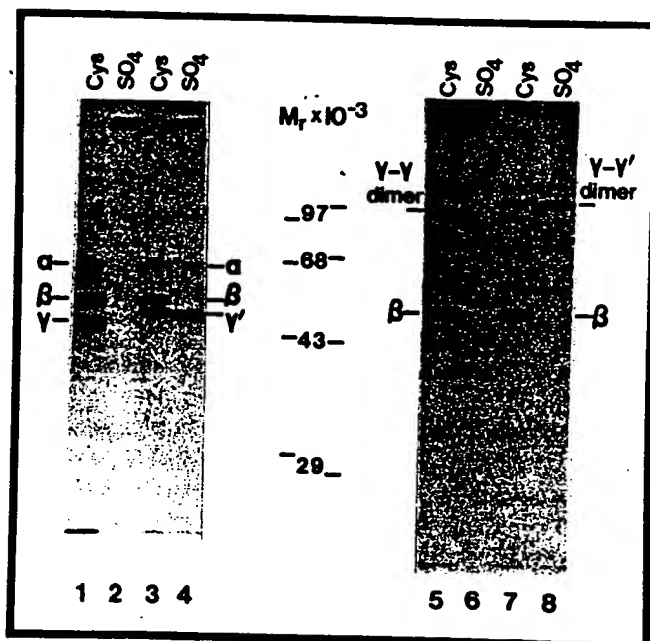


FIGURE 4: [ $^{35}$ S]Cysteine- and [ $^{35}$ SO $_4$ ]-labeled fibrinogens. [ $^{35}$ S]Cysteine- and [ $^{35}$ SO $_4$ ]-labeled fibrinogens from BHK-Fbg and BHK-Fbg $\gamma'$  cells were immunoprecipitated or clotted and run on gels. Lanes 1-4 show recombinant fibrinogens immunoprecipitated and run on 10% reduced gels: (1) [ $^{35}$ S]Cys-labeled BHK-Fbg cells; (2) [ $^{35}$ SO $_4$ ]-labeled BHK-Fbg cells; (3) [ $^{35}$ S]Cys-labeled BHK-Fbg $\gamma'$  cells; (4) [ $^{35}$ SO $_4$ ]-labeled BHK-Fbg $\gamma'$  cells. Lanes 5-8 show recombinant fibrinogens clotted and run on 10% reduced gels: (5) [ $^{35}$ S]Cys-labeled BHK-Fbg cells; (6) [ $^{35}$ SO $_4$ ]-labeled BHK-Fbg cells; (7) [ $^{35}$ S]Cys-labeled BHK-Fbg $\gamma'$  cells; (8) [ $^{35}$ SO $_4$ ]-labeled BHK-Fbg $\gamma'$  cells.

a great deal is known about its structure and function, the unambiguous assignment of many biological functions to specific domains has yet to be made. The expression system presented here provides the capability to study structure/function relationships directly by site-specific mutagenesis. Secreted rFbg was indistinguishable from normal fibrinogen by several criteria. Structurally, unreduced rFbg comigrated

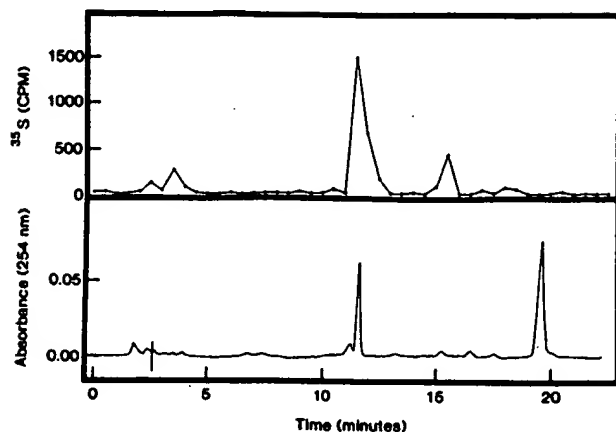


FIGURE 5: Tyrosine *O*-sulfate analysis. In the upper panel,  $^{35}\text{SO}_4$ -labeled rFbg $\gamma'$  was digested with carboxypeptidase Y, derivatized with phenyl isothiocyanate, and chromatographed on a C18 column; 0.5-min fractions were collected and assayed for radioactivity. In the lower panel, Tyr *O*-sulfate standard was derivatized and chromatographed under the same conditions and monitored for absorbance at 254 nm.

with  $M_r$  340 000 HepG2 fibrinogen on polyacrylamide gels. When reduced, the constituent  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chain bands comigrated with their normal counterparts. These results show that rFbg was secreted as the normal six-chain molecule with a stoichiometry of  $(\alpha\beta\gamma)_2$ . Similarly, rFbg $\gamma'$  showed identical  $\alpha$  and  $\beta$  chains upon reduction, with the larger  $\gamma'$  chain migrating more slowly, as expected.

Functionally, the recombinant fibrinogens were active in both clotting and cross-linking. It is significant that the labeled recombinant fibrinogens not only were incorporated into the clot but also were cross-linked by the factor XIIIa transglutaminase activity. This indicates that the recombinant fibrinogens were aligned within the fibrin fibrils in the correct orientation, such that the carboxyl ends of the  $\gamma$  (or  $\gamma'$ ) chains in adjacent fibrin monomers were close enough to one another for cross-linking into dimers; similarly, the  $\alpha$  chains were in the correct orientation to allow cross-linking into a high molecular weight polymer. Thus, by these criteria, the recombinant fibrinogens functioned like normal HepG2 fibrinogen.

An interesting structural difference seen between rFbg and rFbg $\gamma'$  was the sulfation of the  $\gamma'$  chain. An early publication (Jevons, 1963) reported the presence of Tyr *O*-sulfate in human fibrin, which was later localized to the  $\beta$  chain (Liu et al., 1985), on the basis of its mobility on gels. A reexamination of these findings (Hortin, 1989) suggested that the sulfated chain may be the  $\gamma'$  variant, on the basis of its mobility and protease resistance. The present report demonstrates that the  $\gamma'$  chain is indeed sulfated, rather than the closely migrating  $\beta$  chain.

Pronase hydrolysis of the sulfated chain in human fibrinogen from HepG2 cells showed that the sulfation occurred on Tyr residues (Liu et al., 1985; Hortin, 1989). In the BHK cell

expression system, the vast majority of the sulfation was also found on Tyr residues, demonstrating that the expression system performs this posttranslational modification correctly. In rat fibrinogen, one Tyr at position 418 is present in the  $\gamma'$  chain which is absent in the  $\gamma$  chain; this is thought to be the sulfated residue (Hirose et al., 1988). Similarly, the human  $\gamma'$  chain also contains Tyr-418; however, an additional Tyr is also present at position 422 (Figure 6). Tyr-418 follows the consensus pattern for sulfated Tyr residues (Huttner, 1988): an acidic amino acid at position -1 (Glu-417) with at least three acidic amino acids from -5 to +5 (Glu-415, Glu-417, Asp-419) and not more than one basic amino acid from -5 to +5 (none present); the presence of turn-inducing amino acids from -7 to -2 and from +1 to +7 (Pro-413, Pro-423); less than three hydrophobic amino acids from -5 to +5 (Leu-421); and an absence of disulfide-bonding Cys residues or N-linked glycosylation sites from -7 to +7. In contrast, Tyr-422 lacked an acidic amino acid at position -1 (Leu-421) but had five acidic amino acids from -5 to +5 (Glu-417, Asp-419, Glu-424, Asp-425, Asp-426), had one turn-inducing amino acid from +1 to +7 (Pro-423) but lacked one from -7 to -2, had less than three hydrophobic amino acids from -5 to +5 (Leu-421, Leu-427), and lacked disulfide-bonded Cys residues or N-linked glycosylation sites from -7 to +7. On the basis of this analysis, and by analogy with the rat  $\gamma'$  chain, Tyr-418 is probably the sulfated residue. Further biochemical characterization of the Tyr *O*-sulfate, however, is necessary. It was not possible to determine the stoichiometry of sulfation, since the specific activities of the cysteine and sulfate pools within the cell are not known. The determination of the stoichiometry of sulfation will require the purification of rFbg $\gamma'$  and the biochemical analysis of the sulfated Tyr residues. One additional possibility is that the  $\gamma'$ -chain 20 amino acid carboxyl extension is not sulfated itself but merely directs sulfation to another part of the chain. However, the rapid and quantitative release of the sulfated Tyr by carboxypeptidase Y suggests that the sulfated residues were near the carboxyl terminus.

The role of the sulfated Tyr is completely unknown, since the role of the  $\gamma'$  chain is unknown. It is apparent that the  $\gamma'$ -chain extension contributes seven extra negatively charged amino acids in Glu and Asp residues, and possibly one or two additional negative charges in sulfated Tyr residues. The effect of these charges on the fibrinogen molecule is puzzling. It is clear from the present data that they do not prevent incorporation into a fibrin clot, nor do they prevent cross-linking of the  $\gamma'$  chains by factor XIIIa. Previous studies using heterodimeric  $\gamma'$ -containing fibrinogen  $(\alpha\beta\gamma)(\alpha\beta\gamma')$  indicated that these molecules have reduced platelet binding and aggregate platelets less effectively (Peerschke et al., 1986). In addition, intracellular fibrinogen stored in platelet  $\alpha$  granules lacks the  $\gamma'$  chain (Francis et al., 1984; Mosesson et al., 1984). However, these findings only illustrate roles in which  $\gamma'$ -

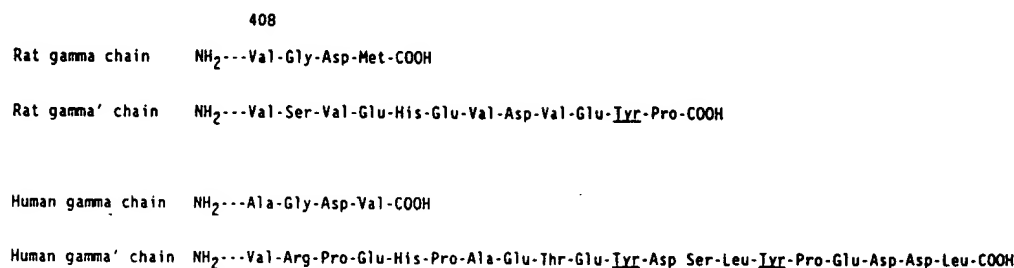


FIGURE 6: Comparison of  $\gamma$ - and  $\gamma'$ -chain carboxyl termini from rat and human fibrinogens. Amino acid sequences for the carboxyl termini of rat and human  $\gamma$  and  $\gamma'$  chains are shown in alignment. The putative Tyr-418 sulfation site in the rat  $\gamma'$  chain is underlined, as is the corresponding site in the human  $\gamma'$  chain and the unique Tyr at position 422.

containing fibrinogen does not participate; the expression system shown in this report should prove useful in the elucidation of its true role in hemostasis.

#### ACKNOWLEDGMENTS

We thank Marie Beltran for technical assistance with the expression of rFbg $\gamma'$ . We also thank Dr. Don Foster for providing the  $\alpha_2$ -antiplasmin expression vector. We express our sincere appreciation to Dr. Stephen Conroy for his assistance with the Tyr O-sulfate analysis and to Dr. Ming-Cheh Liu for his generous gift of the Tyr O-sulfate standard.

Registry No. Tyr, 60-18-4.

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## Expression of biologically active heterodimeric bovine follicle-stimulating hormone in milk of transgenic mice

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Communicated by Neal L. First, June 6, 1991 (received for review April 15, 1991)

**ABSTRACT** Follicle-stimulating hormone (FSH; follitropin) is a pituitary glycoprotein composed of two post-translationally modified subunits, which must properly assemble to be biologically active. FSH has been difficult to purify and to obtain in quantities sufficient for detailed biochemical studies. We have targeted FSH expression to the mammary gland of transgenic mice by using cDNAs encoding the bovine  $\alpha$  and FSH $\beta$  subunits and a modified rat  $\beta$ -casein gene-based expression system. Lines of bigenic mice expressing both subunits have been generated either by coinjection of the subunit transgenes or by mating mice that acquired and expressed transgenes encoding an individual subunit. Up to 60 international units (15  $\mu$ g) of biologically active FSH per ml was detected in the milk of the bigenic mice. These lines provide a model system for studying the post-transcriptional mechanisms that effect the expression and secretion of this heterodimeric hormone.

Follicle-stimulating hormone (FSH; follitropin) is a member of the glycoprotein family of pituitary hormones, which includes thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and chorionic gonadotropin (CG). Like LH and CG, FSH is a gonadotropin and is composed of a common  $\alpha$  subunit that is noncovalently linked to a hormone-specific  $\beta$  subunit (1, 2). FSH has been difficult to purify and to obtain in sufficient quantities for detailed biochemical studies (for a review, see ref. 3). The  $\alpha$  and FSH $\beta$  subunits are post-translationally modified, and the nature and extent of such modifications can exert a profound effect on subunit assembly, secretion, and stability (4-6). Only heterodimers with appropriately glycosylated subunits exhibit significant biological and receptor-binding activity (5, 7, 8). Targeting FSH to the mammary gland of transgenic animals would, therefore, serve as a model system in which to study glycoprotein processing and secretion as well as a means to produce large quantities of FSH. A standardized source of recombinant FSH would be useful to both human and livestock fertilization programs to achieve the reproducible development of ovarian follicles.

Several different milk protein-based constructs have been employed to express diverse heterologous proteins in the milk of a variety of transgenic animals (for reviews, see refs. 9-11). We have demonstrated previously that a -524/+490 minimal rat  $\beta$ -casein promoter fragment can direct the expression of chloramphenicol acetyltransferase to the mammary gland (12). To determine whether the mammary gland could be used to secrete large quantities of a bioactive heterodimeric protein into milk, we have used a modified rat  $\beta$ -casein-based vector to target and express bovine FSH (bFSH) to the mammary gland and into the milk of transgenic mice.

## MATERIALS AND METHODS

**Construction of the Transgenes.** The FSH subunit cDNAs were obtained from Genzyme;  $\alpha$  as a 730-base-pair (bp) *EcoRI* fragment and FSH $\beta$  as a 560-bp *EcoRI/BamHI* fragment. The cDNA fragments were inserted into pUC19 (13) with the rat  $\beta$ -casein -524/+490 fragment (12) and an 850-bp *EcoRI* fragment carrying the simian virus 40 small tumor antigen intron with transcript cleavage and polyadenylation signals (kindly provided by S. Berget, Baylor College of Medicine). A 408-bp *HindIII* fragment of the mouse mammary tumor virus long terminal repeat (LTR) carrying four glucocorticoid response element (GRE) sequences (kindly provided by M. Parker, Imperial Cancer Research Fund Laboratories) was placed at -330 in the rat  $\beta$ -casein fragment of the  $\alpha$  construct.

**Production and Screening of Transgenic Mice.** Transgenic mice were generated and mouse tail DNA was isolated as described previously (12). The polymerase chain reaction (PCR) was employed to screen for positive transgenic mice. The sequences of the synthetic oligonucleotides used in PCR reactions were as follows (5'  $\rightarrow$  3'): 1, GAGCTTCATCTTC-TCTCTGTCTCCTCCGC; 2, ACAGAGACAAATGGCCA-GAATGAC; 3, GCTTTATTGCTTTTCTCCTTATCCT; 4, TCTCTGTAGGTAGTTTGTCCAATTA; 5, AGGCATTC-CACCACTGCTCCCATTCATC; 6, AAAAGGAAACA-GAACTGGACAGACT; and 7, TACTGACCTCTGCTCTC-CGACGGAT.

Transgene cointegration was analyzed by Southern blotting tail DNA (10  $\mu$ g) digested with *EcoRI*. Blots were hybridized with <sup>32</sup>P-labeled  $\alpha$ - or FSH $\beta$ -specific probes prepared by random oligonucleotide labeling.

**RNA Isolation and Analysis.** Total RNA was isolated from mouse mammary gland tissue by the method of Chirgwin *et al.* (14). For Northern blots, RNA (20  $\mu$ g) was fractionated in agarose gels containing formaldehyde (15). For slot blots, RNA (1, 2, or 4  $\mu$ g) was applied to ZetaProbe membrane (Bio-Rad) and compared to known amounts of the  $\alpha$  or FSH $\beta$  cDNAs included as standards. Quantitation was performed by scanning with an LKB laser densitometer.

**Collection of Mouse Milk.** Mice were anesthetized with 1 ml of Avertin (20 mg/ml) administered i.p. immediately prior to milking, and 0.5 ml of oxytocin [200 international units (IU)/ml; Sigma] was administered i.p. before milk samples were harvested by gentle suction into tubes at 4°C. The whey fraction was prepared by centrifugation of skim milk at 16,000  $\times g$  for 15 min at 4°C.

Abbreviations: FSH, follicle-stimulating hormone (follitropin); bFSH, bovine FSH; rbFSH, recombinant bFSH; oFSH, ovine FSH; GRE, glucocorticoid response element; IU, international units.

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**Characterization of FSH in Mouse Milk.** A heterologous double-antibody radioimmunoassay (RIA) was performed as described (16). For immunoblot analysis, whey protein (300  $\mu$ g) was fractionated by SDS/PAGE at room temperature (15). Samples were not heated and did not contain 2-mercaptoethanol. Ovine FSH (oFSH; NIADDK-oFSH-16; 20 National Institutes of Health units/mg) was added to nontransgenic mouse milk for positive controls. A sample of recombinant bFSH (rbFSH) made in Chinese hamster ovary (CHO) cells (a gift of Genzyme) was used to assess the cross-reactivity of the antibody in this assay. Blots were probed with the JAD-17-689 antiserum (16) (1:5000), kindly provided by J. Dias (State of New York Department of Health), and developed with a goat anti-rabbit IgG-horseradish peroxidase enhanced chemiluminescence (ECL) detection scheme (Amersham).

For radioreceptor assays, samples were initially diluted with an equal volume of assay buffer (100 mM Tris-HCl/100 mM sucrose/5 mM  $MgCl_2$ /0.1% bovine serum albumin, pH 7.4) and incubated with a chicken testis receptor preparation (17). The standard was NIH-FSH-S9 (18). Data analysis was by the ALLFIT(FLEXIFIT) program, version 2.6 (Laboratory of Theoretical and Physical Biology, National Institute of Child Health and Human Development). Calculation of the ng of rbFSH was based on the specific activity of bFSH (19). Calculation of FSH activity was from the  $ED_{50}$  of the assay. Acid-dissociation radioreceptor assay experiments (20) measured FSH activity in 50  $\mu$ l of milk surviving 1 M propionic acid treatment for 1 hr at 37°C. Precipitated casein and other milk proteins were removed by centrifugation. The granulosa cell bioassay and chromatofocusing analysis were performed as described (5, 21).

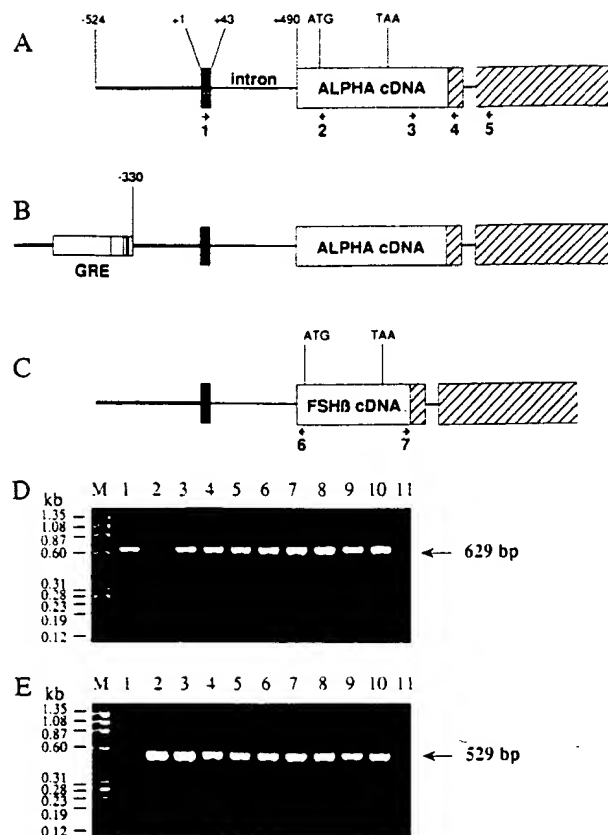
## RESULTS AND DISCUSSION

**Characterization of the  $\alpha$  and FSH $\beta$  Transgenes.** The cDNAs encoding  $\alpha$  or FSH $\beta$  were placed into a rat  $\beta$ -casein expression vector (Fig. 1). Lines of transgenic mice were generated by either individual or coinjection (12, 22) of  $\alpha$  and FSH $\beta$  constructs. A construct carrying four copies of a GRE from the mouse mammary tumor virus promoter (Fig. 1B) was also employed to direct high-level  $\alpha$ -subunit expression, since in the normal pituitary  $\alpha$  is expressed in excess over the dimeric hormone (23–27). Screening by PCR identified founder animals carrying either the  $\alpha$  or the FSH $\beta$  construct or both (Fig. 1D and E).

Southern blot experiments were used to characterize the architecture of the integrated transgenes. Since the transgenes carry a single *Eco*RI site, the detection of strongly hybridizing species in the 2- to 3-kb range (Fig. 2A and B) is diagnostic for transgene cointegration. When the  $\alpha$  or FSH $\beta$  transgenes were coinjected, multiple copies were found to be cointegrated in >85% of the positive lines. Only a few lines carried individual transgenes [e.g., line 7905 carries a single  $\alpha$  transgene (Fig. 2A and B, lane 1)]. Some head-to-head and tail-to-tail cointegration events occurred. Divergent PCR confirmed the head-to-tail orientation (Fig. 2C).

**Expression of  $\alpha$  and FSH $\beta$  mRNAs in Mammary Glands of Lactating Transgenic Mice.** Northern blot analysis indicated the presence of major 1519-nucleotide  $\alpha$  and 1340-nucleotide FSH $\beta$  mRNA species (Fig. 3) corresponding to the expected transcript sizes. The smaller  $\alpha$  mRNA species (Fig. 3A, lane 3) may arise from cleavage and polyadenylation at signals within the 3' untranslated region of the  $\alpha$  cDNA (28, 29). Examination of the transcripts by reverse transcriptase-mediated PCR indicated that most  $\alpha$  and FSH $\beta$  mRNA species encode unit-length proteins (N.M.G. and J.M.R., unpublished results).

When Northern blots were rehybridized with a mouse  $\beta$ -casein exon 7 probe, the  $\beta$ -casein mRNA level was found to be  $\approx$ 5- to 10-fold greater than that observed for the  $\alpha$ -subunit



**FIG. 1.** Structure of the rbFSH transgenes and identification of transgenic mice by PCR. (A) Structure of the  $\alpha$  cDNA transgene. The elements are -524 to +1, the 5' flanking region of rat  $\beta$ -casein; +1 to +43, the noncoding first exon of rat  $\beta$ -casein and 5' splice donor; +490, the 3' splice acceptor and unique *Eco*RI site; ATG and TAA, the bovine  $\alpha$  cDNA open reading frame; hatched region, simian virus 40 small tumor antigen splice and polyadenylation signals. The primers used for PCR were 1, 2, 3, 4, and 5 (see *Materials and Methods*). (B) Structure of the GRE-enhanced  $\alpha$  cDNA transgene. The four GREs are denoted by thin vertical bars. (C) Structure of the FSH $\beta$  cDNA transgene. ATG and TAA denote the bFSH $\beta$  cDNA open reading frame. The primers used for PCR were 6 and 7. (D) PCR analysis for the  $\alpha$  transgene. Primers 1 and 2 were used to screen tail DNA by PCR. Lanes 1–10 represent founder mice 7905, 7502, 2038, 7398, 7485, 7919, 7389, 7667, 7668, and 7904, respectively. Lane 11, nontransgenic mouse control. The sizes of the PCR products are shown on the right and the migration of the DNA markers (lane M) is shown on left in kilobases (kb). (E) PCR analysis for the FSH $\beta$  transgene. Primers 1 and 6 were used to screen tail DNA by PCR. Lanes as in D.

mRNA (data not shown). Since  $\beta$ -casein mRNA has been estimated to make up  $\approx$ 20% of the total mRNA at day 10 of lactation, the level of the  $\alpha$ -subunit mRNA should correspond, therefore, to  $\approx$ 2% of the total mRNA, in agreement with the quantitative slot blot determination (see below).

Two-thirds of the mice carrying the GRE-enhanced  $\alpha$  construct expressed the transgene, whereas only one-sixth of those lacking the GRE expressed  $\alpha$ . Of mice carrying minimal  $\alpha$  and FSH $\beta$  constructs, 3 of 11 (27%) expressed both transgenes, while 6 of 10 (60%) expressed the cointegrated GRE $\alpha$  and FSH $\beta$  constructs. Lines of transgenic mice carrying the GRE-enhanced constructs expressed more frequently (30, 31) and at higher levels (see below). Line 7905 (single copy of GRE $\alpha$ ) has been bred to line 7502 (4 to 6 copies of a tandemly arranged FSH $\beta$ ) to establish line 2038 (Fig. 1D and E), which expressed both independent loci (Fig. 3).

**Secretion of rbFSH into Mouse Milk.** rbFSH was detected in milk collected at lactation (Fig. 4A), but not in milk from nontransgenic littermates, by using a heterologous double-

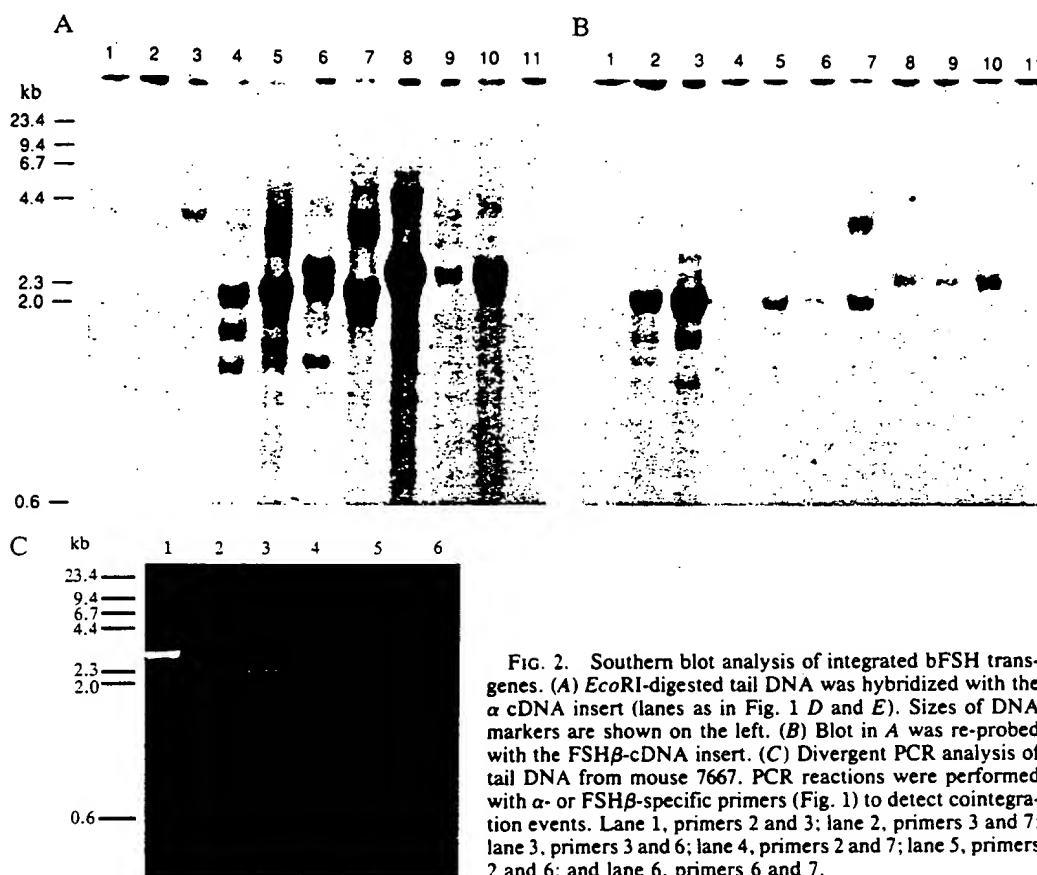


FIG. 2. Southern blot analysis of integrated bFSH transgenes. (A) *EcoRI*-digested tail DNA was hybridized with the  $\alpha$  cDNA insert (lanes as in Fig. 1 D and E). Sizes of DNA markers are shown on the left. (B) Blot in A was re-probed with the FSH $\beta$ -cDNA insert. (C) Divergent PCR analysis of tail DNA from mouse 7667. PCR reactions were performed with  $\alpha$ - or FSH $\beta$ -specific primers (Fig. 1) to detect cointegration events. Lane 1, primers 2 and 3; lane 2, primers 3 and 7; lane 3, primers 3 and 6; lane 4, primers 2 and 7; lane 5, primers 2 and 6; and lane 6, primers 6 and 7.

antibody RIA. Proteins present in normal mouse milk did not interfere with the assay.

The species of FSH present in milk were further characterized by immunoblotting (Fig. 4B). Preparations of pituitary oFSH (lanes B, C, and D in Fig. 4B) and rbFSH prepared from transfected CHO cells (lane I in Fig. 4B) were included as controls. The antiserum to oFSH detected a species of  $\approx$ 38 kDa in the milk from bigenic mouse 8942 (lanes E and K in Fig. 4B). The 38-kDa species corresponds in size to the species detected in both the oFSH and rbFSH standards. Some microheterogeneity in the post-translational modifications of the FSH may explain the broad bands observed (see chromatofocusing results) (32, 33). One microgram of the oFSH standard (lane B) gave a much stronger signal at 38 kDa than an equivalent amount of the CHO rbFSH protein (lane

I), reflecting that the antiserum was raised against oFSH rather than bFSH.

A strongly immunoreactive species with a mass of 18 kDa was detected in the milk from bigenic mice, as well as from mice expressing only the  $\alpha$ -subunit mRNA and may be free  $\alpha$  subunit. This was not detected in the control milk sample. The immunoblot and RIA results suggest the polyclonal anti-oFSH antiserum can crossreact with both heterodimer and free  $\alpha$  subunit, and it may contain species recognizing free bovine  $\alpha$  and heterodimer, but with different affinities. Therefore, the immunoblot could not be used to quantitatively determine the relative abundance of  $\alpha$  and rbFSH.

Steady-state  $\alpha$  and FSH $\beta$  mRNAs were quantitated by slot blot hybridization analysis. Summarized in Table 1,  $\alpha$  mRNA levels were consistently higher, 7- to 17-fold, than those for FSH $\beta$  mRNA. Levels of both mRNAs were independent of transgene copy number; line 7905 carries a single GRE $\alpha$  transgene yet expresses high levels of  $\alpha$  mRNA. Consistent with previous results, expression appeared to be highly dependent on the site of integration (11, 12, 31), and the level of mRNA was observed to vary as much as 3-fold between littermates (e.g.,  $\alpha$ -FSH mRNA, line 7919). The relatively high  $\alpha$  and low FSH $\beta$  mRNA levels suggest that post-transcriptional mechanisms influence their steady-state levels, supporting the hypothesis that the 3' untranslated region of FSH $\beta$  mRNA may impart instability (34).

Milk of bigenic mouse 8942 was capable of displacing an  $^{125}$ I-labeled purified porcine FSH preparation from chicken testis FSH receptors (Fig. 4C) (2, 17). No displacement was observed when milk from a nontransgenic littermate was used. The calculated competitive binding displacement (single point assay, Fig. 4C) of milk treated with 1 M propionic acid was equivalent to only 82 ng of FSH as compared to 2000 ng of FSH in 50  $\mu$ l of untreated milk, representing 96% inactivation.

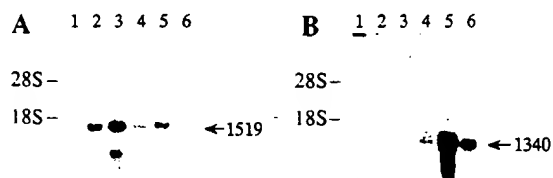


FIG. 3. Northern analysis of FSH mRNA from transgenic mice at day 10 of lactation. (A) Hybridized with the  $\alpha$  cDNA insert. Lane 1, RNA prepared from a nontransgenic mouse; lanes 2-6, RNA from mouse (line number) 9667 (7905), 9415 (7398), 8611 (7667), 2038 (2038), and 9434 (7502), respectively. The relative migration of the 18S and 28S ribosomal RNAs is shown on the left. Length in nucleotides is given on the right. Time of autoradiography was 30 min. (B) Hybridized with the FSH $\beta$  cDNA insert. Lanes as in A. Time of autoradiography was 20.5 hr.



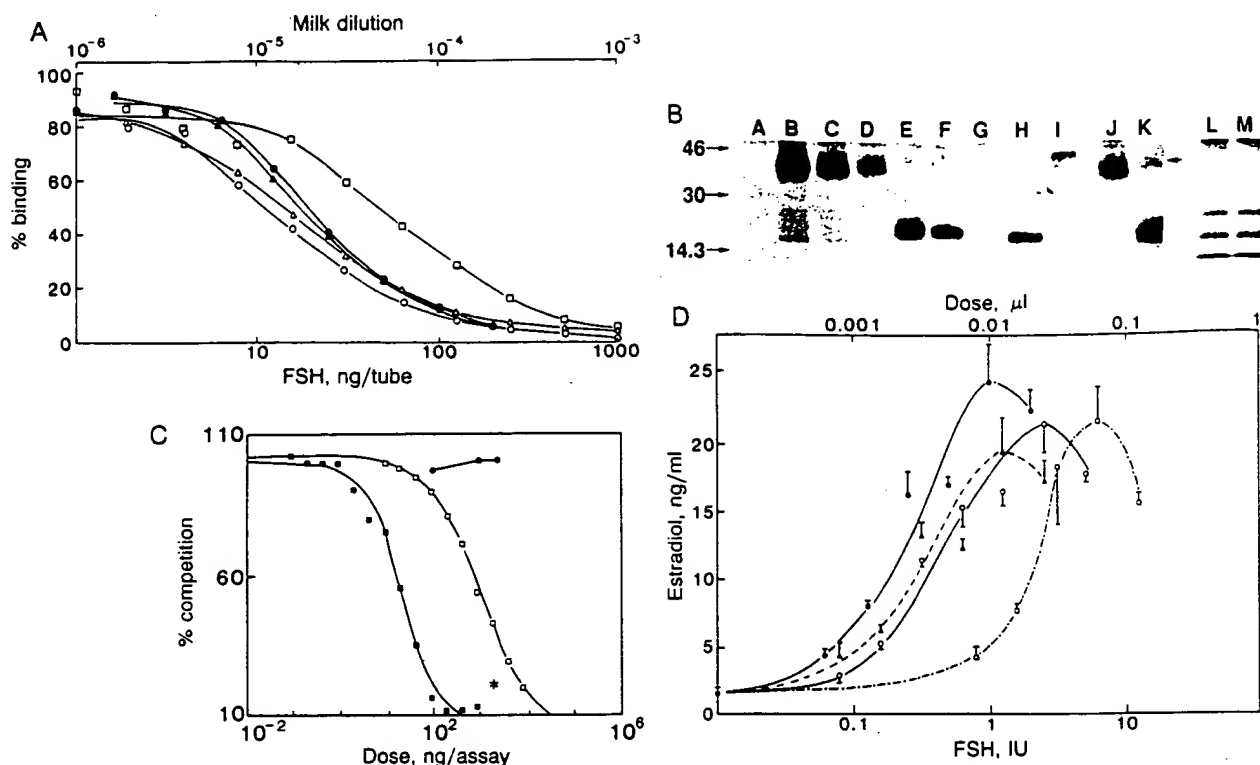


FIG. 4. Characterization of rbFSH in milk of transgenic mice. (A) RIA using rabbit antiserum JAD-17-679. Standard curves: (●), USDA B5 standard; (▲), USDA B5 standard in nontransgenic milk. Sample inhibition curves are (values obtained for FSH in milk calculated from the dilution giving 50% inhibition in parentheses): (○), milk 8942 (2.3 mg/ml); (Δ), milk 8611 (2.5 mg/ml); (□), milk 1262 (0.63 mg/ml). (B) Immunoblot analysis of FSH in milk. Milk samples fractionated by SDS/PAGE were probed with JAD-17-679. Lanes A–D, normal mouse milk with 0, 1, 0.5, 0.25  $\mu$ g of oFSH added, respectively. Lanes E–H are milk from mice (line number in parentheses) 8942 (7919), 9667 (7905), 9434 (7502), and 2038 (2038), respectively. Lane I, CHO rbFSH (200  $\mu$ g of total protein; 1.25  $\mu$ g of FSH). Lanes J and K are 2-hr exposures of lanes D and E. Lanes L and M show lanes equivalent to D and E from a gel run in parallel and stained with Coomassie blue. (C) Competitive binding experiments for rbFSH in milk, using a chicken testis radioreceptor assay. Samples used were FSH (NIH-FSH-S9; □), milk from transgenic mouse 8942 (■), and milk from a nontransgenic mouse (●). The putative ng of rbFSH has been plotted in comparison with the ng of NIH-FSH-S9 used in the assay (to avoid the weight-to-dilution comparison). A comparable dilution for the control milk (●) is shown on the same scale. A sample of milk 8942 treated in 1 M propionic acid for 1 hr at 37°C (\*) was also analyzed. (D) Analysis of bioactive rbFSH by granulosa cell aromatase assay. Granulosa cell cultures were treated with increasing aliquots of milk whey protein fractions from transgenic and control mice. Symbols as in A; data are mean  $\pm$  SEM.

These results, summarized in Table 1, indicate that the rbFSH secreted into milk can interact with FSH receptors.

To measure the biological activity of the rbFSH, rat granulosa cell *in vitro* bioassays were utilized (Fig. 4D). In granulosa cells, FSH stimulates both the conversion of cholesterol to pregnenolone and the aromatization of the

estrogen precursor androstenedione (33). The results are summarized in Table 1. Milk samples from independent bigenic lines (mice 7994, 1262, 8611, and 8942) contained high levels of biologically active FSH. High FSH activity was detected in milk from line 2038, while milk from the parental lines contained no detectable bioactive FSH. Therefore,

Table 1. Summary of FSH subunit mRNA levels in the mammary gland and FSH activity in the milk of lactating transgenic mice

Line	Mouse	Construct	ng $\alpha$ mRNA/ $\mu$ g total RNA (A)	ng FSH $\beta$ mRNA/ $\mu$ g total RNA (B)	A/B ratio	FSH, IU/ml	
						Radioreceptor assay	Granulosa bioassay
ICR			<0.1	<0.1	ND	<0.1 (n = 1)	<0.1 (n = 1)
7905	9667	GRE $\alpha$	10.8	<0.1	ND	ND	<0.1 (n = 1)
7502	9434	FSH $\beta$	<0.1	0.3	ND	ND	<0.1 (n = 1)
7398	7994	$\alpha$ FSH $\beta$	3.8	0.2	17.3	ND	39.2 (n = 1)
7485	1262	$\alpha$ FSH $\beta$	1.4	0.2	7.3	ND	10.6 $\pm$ 4.2 (n = 6)
7667	8611	GRE $\alpha$ FSH $\beta$	2.8	0.2	12.6	ND	39.2 $\pm$ 15.5 (n = 4)
7919	7919	GRE $\alpha$ FSH $\beta$	2.3	0.4	5.6	ND	ND
7919	8941	GRE $\alpha$ FSH $\beta$	2.8	0.3	10.7	ND	ND
7919	8942	GRE $\alpha$ FSH $\beta$	6.6	0.5	13.6	66.7 (n = 1)	66.2 $\pm$ 14.8 (n = 5)
2038	2038	GRE $\alpha$ $\times$ FSH $\beta$	8.2	0.7	11	ND	36 (n = 1)

Values in columns A and B are rounded off. The A/B ratio is accurate to two significant figures. FSH subunit mRNA levels are expressed as ng of specific mRNA per  $\mu$ g of total RNA per assay. To assay FSH in milk, samples harvested from lactating female mice were characterized by the radioreceptor and *in vitro* granulosa cell bioassays; results are given  $\pm$  SEM. ND, not determined.

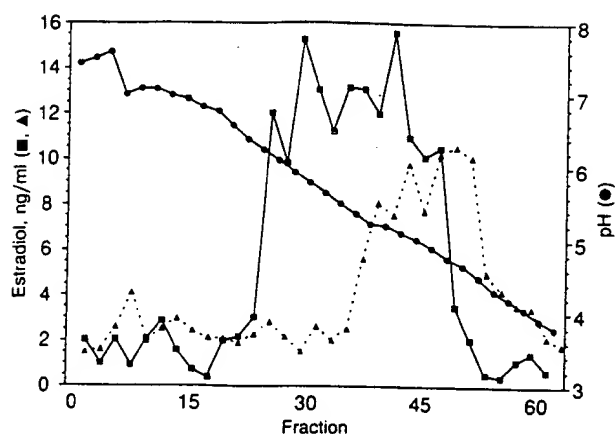


FIG. 5. Analysis of rbFSH by chromatofocusing. Samples of rbFSH from bigenic mouse milk or transfected CHO cells (Genzyme) were chromatographed on a PBE-94 column (Pharmacia). The pH (●) and ability to stimulate estrogen synthesis by 8942 milk (■;  $\approx 800$  ng of FSH) and CHO FSH (▲;  $\approx 2.5$   $\mu$ g of FSH) were determined from alternate fractions.

production of estrogen in the granulosa cell assay was specifically related to the presence of heterodimeric FSH. The amounts of rbFSH in milk from mouse 8942 determined by the radioreceptor and granulosa cell assays were quite similar. This value of 66 IU/ml corresponds to  $\approx 15.3$   $\mu$ g of FSH per ml, assuming 1  $\mu$ g of FSH = 4 IU of FSH (19). No adverse reproductive consequences were observed in the expressing bigenic lines, and rbFSH was not detected in the serum collected from lactating animals (D. Bolt, personal communication), suggesting that rbFSH is secreted vectorially into milk. Normal patterns of transgene transmission and expression have been observed in several litters of offspring for all our expressing lines.

Since isoforms of FSH can be separated on the basis of their isoelectric properties, which are, in part, related to terminal sialic acid content (32, 33) chromatofocusing analysis was performed. Fractions from 8942 milk (4 IU total) and the CHO rbFSH (10 IU total) were collected, the pH was measured, and FSH activities were determined by granulosa cell bioassay. The 8942 rbFSH had one major peak of activity between pH values 6.1 and 4.2 (Fig. 5) and the CHO rbFSH profile exhibited a similar single peak (pH 5.2 to 4.0). Both profiles are consistent with the observations of Galway et al. (5) for FSH with appropriate N-linked carbohydrate structures. The broader peak observed for transgenic rbFSH probably reflects the capacity of the mammary gland to add terminal sialic acid residues to these proteins. The lack of terminal sialic acid residues does not affect FSH receptor binding or *in vitro* bioactivity but may, however, be related to enhanced clearance rates for FSH in blood plasma (5, 32).

By several independent criteria we have demonstrated that rbFSH can be produced in transgenic mice using a rat  $\beta$ -casein expression system. As post-transcriptional mechanisms are probably responsible for the differences observed in the relative levels of the  $\alpha$  and FSH $\beta$  subunit mRNAs, it may be possible with appropriate engineering to express a more stable FSH $\beta$  mRNA, thereby increasing the levels of rbFSH in milk. For example, a "second generation" of transgenes has been constructed to determine whether higher steady-state levels of FSH $\beta$  mRNA will result from the precise exchange of FSH $\beta$  and  $\alpha$  cDNA open reading frames. The lines of mice bearing the individual  $\alpha$ , and FSH $\beta$  transgenes provide useful models for the study of the mechanisms regulating the post-translational processing of both the individual subunits and the heterodimer. Finally, these

studies have demonstrated that the mammary gland can be used as a bioreactor to direct the high expression and vectorial secretion into milk of heterodimeric proteins requiring extensive post-translational modifications. Although the quantities of glycosylated hormone produced in mice are sufficient for further biochemical analysis, the introduction of such transgenes into livestock (9) will be required to provide sufficient quantities for both research and commercial purposes.

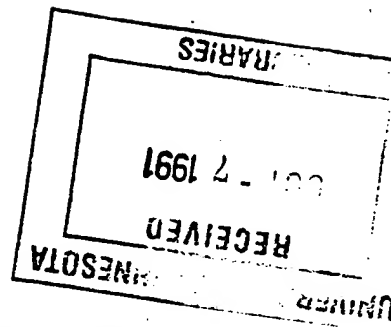
We thank T. Duffy, F. Farzam, C. Nichols, and Hyun Nahm for technical assistance. Hormone standards were gifts of the U.S. Department of Agriculture Animal Hormone Program. This work was supported in part by the U.S. Department of Agriculture (Grant 88-37266-3951 to J.M.R.) and Granada Biosciences (J.M.R.) and the National Institutes of Health (Grant DK-09801 to D.N.W.).

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OCTOBER 1, 1991

VOLUME 88

NUMBER 19



# Proceedings OF THE National Academy of Sciences

OF THE UNITED STATES OF AMERICA



Proceedings  
OF THE  
National Academy  
of Sciences  
OF THE UNITED STATES OF AMERICA

October 1, 1991

Volume 88, Number 19

pp. 8283–8864

Table of Contents

CONDENSED INFORMATION FOR CONTRIBUTORS

i

AUTHOR INDEX

xi–xiii

*Physical Sciences*

CHEMISTRY

*In vivo* catalysis of a metabolically essential reaction by an antibody

Ying Tang, James B. Hicks, and Donald Hilvert

8784–8786

Studies on DNA-cleaving agents: Computer modeling analysis of the mechanism of activation and cleavage of dynemicin–oligonucleotide complexes

Paul A. Wender, Robert C. Kelly, Suzanne Beckham, and Benjamin L. Miller

8835–8839

MATHEMATICS

Theory of symmetry classes

N. Metropolis, Gian-Carlo Rota, and Joel A. Stein

8415–8419

*Biological Sciences*

BIOCHEMISTRY

Mechanism of synergistic transcriptional transactivation by the human glucocorticoid receptor

A. P. H. Wright and J.-Å. Gustafsson

8283–8287

# Expression of a Whey Acidic Protein Transgene during Mammary Development

## EVIDENCE FOR DIFFERENT MECHANISMS OF REGULATION DURING PREGNANCY AND LACTATION\*

(Received for publication, September 27, 1990)

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Expression of the mouse whey acidic protein (WAP) gene is specific to the mammary gland, is induced several thousand-fold during pregnancy, and is under the control of steroid and peptide hormones. To study developmental regulation of the mouse WAP gene, a 7.2-kilobase (kb) WAP transgene, including 2.6 kb of 5'- and 1.6 kb of 3'-flanking sequences, was introduced into mice. Of the 13 lines of mice examined, 6 expressed the transgenes during lactation at levels between 3 and 54% of the endogenous gene. Although expression was dependent on the site of integration, the transgenes within a given locus were expressed in a copy number-dependent manner and were coordinately regulated. The WAP transgenes were expressed specifically in the mammary gland, but showed a deregulated pattern of expression during mammary development. In all six lines of mice, induction of the WAP transgenes during pregnancy preceded that of the endogenous gene. During lactation, expression in two lines increased coordinately with the endogenous gene, and in three other lines of mice, transgene expression decreased to a basal level. These data indicate that the 7.2-kb gene contains some but not all of the elements necessary for correct developmental regulation. At a functional level it appears as if a repressor element, which inactivates the endogenous gene until late pregnancy, and an element necessary for induction during lactation are absent from the transgene. Complementary results from developmental and hormone induction studies suggest that WAP gene expression during pregnancy and lactation is mediated by different mechanisms.

Expression of milk protein genes is dependent upon interactions between tissue-specific and developmentally and hormonally induced regulatory factors (1). Our laboratory uses the mouse whey acidic protein (WAP)<sup>1</sup> gene as a model to study the structure and function of the corresponding regulatory elements. The WAP gene encodes the major whey

protein in mice (2), rats (1), and rabbits (3) and is expressed almost exclusively in the mammary gland. The steady state level of mouse WAP mRNA increases several thousand-fold between the virgin state and mid-lactation (4, 5). This induction depends upon the presence of lactogenic hormones, glucocorticoids, and insulin (5) but may also require some, as yet undefined, features of cell-cell interactions occurring within the mammary gland (6).

WAP gene expression increases sharply between day 15 and 17 of pregnancy (5), a period during which the levels of placental lactogens are near maximal but prolactin levels are low (7). Insulin and hydrocortisone are present throughout pregnancy. In contrast to WAP the  $\beta$ -casein gene is induced at day 10 of pregnancy (8), coincident with the increase in placental lactogens (7). In spite of their different temporal patterns of expression during pregnancy, induction of both the WAP and  $\beta$ -casein genes in organ explant cultures from mid-pregnant mice requires all three hormones, insulin, hydrocortisone, and prolactin (5, 8-10). The mechanisms by which these hormones activate milk protein gene expression is unclear. Doppler *et al.* (11) have shown in tissue culture cells transfected with a  $\beta$ -casein gene that prolactin and hydrocortisone act through promoter sequences and therefore presumably on the transcriptional level. However, Rosen and co-workers (12) have evidence that these hormones act predominantly at a post-transcriptional level.

Previous studies have shown that a hybrid gene containing 5'-flanking sequences of the mouse WAP gene is expressed specifically in the mammary gland of transgenic mice (4, 13), suggesting that mammary-specific elements reside in 2.6 kb of the promoter upstream region. However, correct developmental and hormonal regulation of the chimeric genes was not observed (5). To test whether sequences downstream from the promoter are required for correct regulation of the WAP gene, we have introduced into mice a 7.2-kb fragment of DNA which encompasses the entire transcribed region of the mouse WAP gene, 2.6 kb of 5'- and 1.6 kb of 3'-flanking DNA. Analysis of WAP transgene expression in six independent lines of mice allowed us to study the contribution of regulatory elements within the 7.2 kb to the developmental and hormonal regulation of the mouse WAP gene. In addition, the introduction of three different WAP gene alleles into a single integration site allowed us to evaluate the influence of surrounding chromatin on the level of expression and developmental regulation of individual transgenes within one locus.

### MATERIALS AND METHODS

**Recombinant Plasmids**—The plasmid pBS WAP, containing a 7.2-kb EcoRI fragment with the mouse WAP gene (14), was linearized

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§ Supported by a postdoctoral fellowship from the Cystic Fibrosis Foundation.

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<sup>1</sup> The abbreviations used are: WAP, mouse whey acidic protein; kb, kilobase(s); PCR, polymerase chain reaction.

with either *KpnI* (exon 1), *SalI* (exon 3), or *BamHI* (exon 4), blunted, and ligated with either *HindIII*, *SmaI*, or *NdeI* linkers. Three WAP linker alleles were generated: the KH allele contained one *HindIII* linker in the *KpnI* restriction site; the SSM allele carried one *SmaI* linker in the *SalI* restriction site; and the BN allele had five *NdeI* linkers inserted into the *BamHI* restriction site (Fig. 1).

Templates used to generate synthetic RNA transcripts containing both the linker-allele and the corresponding wild-type sequences were constructed by subcloning an exon containing the linker and the equivalent wild-type sequence oligonucleotide into the plasmid pBS. Plasmid pKpH contains the first exon of the KH WAP allele and a copy of the corresponding wild-type sequence; plasmid pSIM contains wild-type and allelic sequences spanning the *SalI* site; and plasmid pBmD contains the wild-type and allelic sequences surrounding the *BamHI* site from the fourth exon. *In vitro* transcription with either T3 or T7 RNA polymerase generated sense transcripts from linearised plasmids.

**Generation of Transgenic Mice**—WAP alleles were separated from vector sequences by digestion with *EcoRI*, followed by electrophoresis in an agarose gel (FMC). DNA fragments were then isolated by electroelution and purification on a ion-exchange column (NACS prep; Bethesda Research Laboratories). The fragments were microinjected into the pronuclei of zygotes obtained from C57BL6/SJL F1 female mice. Two classes of mice were produced, KH mice were generated by injection of the KH allele alone, and KSB mice were produced using an approximately equimolar mixture of the three alleles.

**DNA Analysis**—Transgenic founder mice were identified by Southern blot analysis of tail DNA. DNA was prepared from tail tissue by digestion with proteinase K and precipitation with ethanol. The phenol/chloroform extractions were eliminated without adversely affecting the results of Southern or PCR analysis. Transgenic offspring were produced by breeding the founders with C57BL6/SJL F1 mice. Once a stable line was established, offspring were screened by PCR. Mice carrying the KH allele were identified by amplification of the first WAP exon and testing the product for the presence of a *HindIII* restriction site. Amplification of the fourth WAP exon, which in the case of the BN gene contained an additional 50 base pairs and produced a larger PCR product, was used to identify mice carrying the BN allele.

The copy number of the alleles carried by each line was calculated by quantitative PCR. Pairs of primers flanking the *KpnI*, *SalI*, and *BamHI* restriction sites of the WAP gene were used to amplify the first, third, and fourth exons, respectively, from approximately 100 ng of genomic DNA. The products were denatured with sodium hydroxide dotted onto GeneScreen Plus and hybridized with single-stranded oligonucleotides specific for either the allelic or corresponding wild-type sequence. The hybridizations were carried out as described in the section "RNA Analysis." To account for the different hybridization efficiency and labeling of each oligonucleotide probe, blots also contained a serial dilution of the plasmids pKpH, pSIM, and pBmD. The dots were excised, and the bound radioactivity was quantitated by scintillation counting. Results were normalized against the standard serial dilution of plasmids, and the ratio of allele to wild-type sequences was calculated. The copy number of each allele in a transgenic line was determined, based on these ratios and the results of Southern blots.

**Mammary Explant Culture**—Mammary organ explants were prepared and cultured as described previously (5). Concentrations of hormones in organ culture were 100 ng/ml of insulin and hydrocortisone and 1 µg/ml of prolactin.

**RNA Analysis**—RNA was prepared from fresh mammary tissue or organ explants using guanidine thiocyanate and acid phenol (15), fractionated on a formaldehyde agarose gel, and blotted onto GeneScreen Plus essentially as described (16). Polyadenylated RNA was prepared by a batch method (17). Expression of the WAP gene alleles was analyzed with the aid of the following allele-specific oligonucleotide probes.

KH: 5' GGCAACGCATGCAAGCTTGCGGTGTCAGGCA 3'

SSM: 5' GACACAGTCGACCCCGGGTTCGACGTTGCAG 3'

BN: 5' GTTCTCTCTGGATCCCATATGGCCATATGGC 3'

Oligonucleotides which detected the equivalent sites in the wild-type mRNA were as follows.

KPN: 5' CAACGCATGGTACCGGTGTCA 3'

SAL: 5' TGACACAGTCGACGTTGCAGC 3'

BAM: 5' TTCTCTCTGGATCCAGGAGTG 3'

Hybridizations were performed in 0.4 M NaCl, 1% sodium dodecyl sulfate, 100 mg/ml denatured herring sperm DNA and contained <sup>32</sup>P-labeled oligonucleotide probes at 1 ng/ml. All oligonucleotide probes were end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP as described previously (17). Hybridizations with the allele or wild-type-specific oligonucleotide probes were performed at 65 and 55 °C, respectively. The radioactivity associated with a band on the filter was quantitated by scintillation counting. In order to measure the relative levels of transgene expression, blots included a serial dilution of an equimolar mixture of the three synthetic transcripts generated *in vitro* from plasmids pKpH, pSIM, and pBmD. In addition a standard sample of RNA obtained from a nontransgenic lactating mouse was included on blots to demonstrate the specificity of the oligonucleotides. Based on the serial dilution of synthetic RNAs, signals obtained with the different allele-specific oligonucleotides were normalized and expression levels of alleles could be compared against each other or against the endogenous gene when applicable.

The mouse  $\beta$ -casein probe was a 67-mer oligonucleotide specific for the leader peptide. The mouse keratin 18 probe was generated by random priming of a cDNA insert prepared from plasmid pUC97B (18), that had been kindly provided by Dr. Robert Oshima.

## RESULTS

**Generation of Mice Carrying Mouse WAP Transgenes**—To distinguish between the endogenous and transgenic mouse WAP mRNAs, we tagged the transgenes by inserting linkers into either exons 1, 3, or 4 of the mouse WAP gene. Three linker alleles were produced: the KH allele contained a *HindIII* linker in the *KpnI* site; the SSM allele had a *SmaI* linker at the *SalI* site; and the BN allele carried five *NdeI* linkers at the *BamHI* site (Fig. 1). The 0.65-kb WAP mRNA transcribed from the different WAP gene alleles was detected by hybridization with antisense oligonucleotides specific for the linker insertions.

Two classes of transgenic mice were produced; KH mice, which carried KH alleles, and KSB mice, which contained all three alleles. Transgenic mice containing the three alleles were generated to evaluate whether insertion of any of the linker molecules would interfere with gene expression. From five KH and fifteen KSB founder mice, thirteen lines of mice transmitted the transgenes to their offspring. Of these founder mice, three KH (3350A, 3350B, 3350C) and four KSB lines

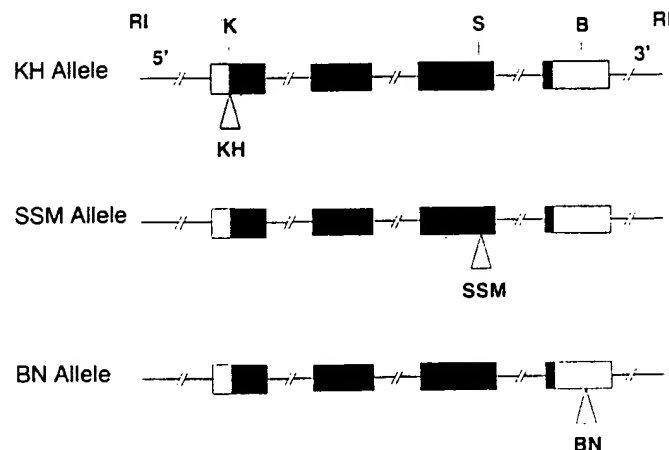


FIG. 1. Structure of the WAP gene alleles. A 7.2-kb genomic fragment spanning the WAP gene was modified by the insertion of oligonucleotide linkers into either the *KpnI* (K), *SalI* (S), or *BamHI* (B) restriction sites, producing three different alleles, KH, SSM, and BN, respectively. The open and closed boxes represent untranslated and translated regions of the four WAP gene exons, respectively.

(3336, 3441, 3628, 3631) expressed the WAP transgenes during lactation at levels between 3 and 54% of the endogenous WAP gene. Expression was confined to the mammary gland (data not shown). A comparison of WAP transgene copy number and expression between six of these lines indicated that the transgene was expressed in a position dependent and copy number independent manner (Table I). Apparently the 7.2-kb WAP transgene does not represent a complete regulatory unit. However, expression levels of this construct were at least an order of magnitude higher than of hybrid genes containing just the WAP gene promoter (4, 13), suggesting the presence of additional regulatory elements downstream of the promoter.

Since the three WAP gene alleles cointegrated in KSB mice in a tandem head-to-tail arrangement (data not shown), we were able to investigate whether expression of the transgenes, which was sensitive to the surrounding chromatin (Table I), was also subject to position effects within the transgene locus. The copy number for each transgenic allele within the loci of the KSB lines was determined by quantitative PCR and was correlated with the steady state levels of the different RNA transcripts (Table II). In the three lines tested, expression of the WAP SSM and BN transgene alleles was proportional to their copy number within the transgene array, suggesting that alleles within a locus are equally active. However, the two copies of KH alleles of line 3336 were only expressed at 50% of the other alleles, and the single copy of KH allele in line 3441, situated at the border of the transgene cluster as judged

by Southern analysis (data not shown), was also expressed at significantly lower levels than the SSM and BN alleles. Taken together the results suggest that most of the WAP transgenes within a transgene locus were expressed and that the level of expression among them was similar. Nevertheless, consistently lower expression of KH transcripts in different lines may indicate that the *HindIII* linker did interfere quantitatively with expression of the transgene.

**Regulation of WAP Transgene Alleles during Development—**RNA prepared from the mammary glands of female transgenic mice at various stages of development, from virgin through pregnancy and lactation, was analyzed on replicate Northern blots for either the endogenous WAP mRNA or transcripts from the different WAP transgene alleles. The endogenous gene was induced between 13 and 17 days of pregnancy and expression peaked around mid-lactation (Figs. 2 and 3). However, the expression of WAP transgenes differed qualitatively and quantitatively from the endogenous gene (Fig. 2). The developmental patterns obtained with lines 3350B and 3336 illustrate two general aspects of WAP transgene expression. First, although the absolute levels of transgene expression differed between lines of mice, the patterns of induction during pregnancy were similar. Transgenic WAP mRNA was detected at day 13 of pregnancy, preceding induction of the

TABLE I  
Comparison between WAP transgene copy number and transgene expression in six independent lines of mice

Transgene copy number (per diploid genome) was determined by quantitative PCR. Transgene expression was quantitated by Northern analysis of total RNA prepared from mice at day 2 of lactation hybridized with oligonucleotides specific for either the endogenous or transgenic WAP gene (WT).

Line	Copy No.	Expression of WT
		%
3350 A	11	45
3350 B	15	54
3350 C	7	3
3336	12	46
3628	20	34
3441	14	18

TABLE II  
Expression of different WAP transgene alleles within a single locus

Expression of WAP alleles in mice of three KSB lines at day 17 of pregnancy (P17) and day 2 of lactation (L2) were quantitated by Northern blots and divided by the number of copies of the allele (per cell) present in the line.

Line	Stage	Allele		
		KH	SSM	BND
cpm/copy				
3336	P17	2 <sup>a</sup>	8	2
		84	184	154
		188	349	308
3441	P17	1	5	8
		42	172	124
		30	246	256
3628	P17	ND <sup>b</sup>	88	93
		1	10	9
		ND	86	99
	L2	ND	313	332
		ND	534	510

<sup>a</sup> Indicates copy number.

<sup>b</sup> ND, not determined.



FIG. 2. Analysis of endogenous and transgenic WAP RNA during mammary development. Ten  $\mu$ g of total RNA prepared from the mammary glands of virgin mice (V) and at various days during pregnancy (P) and lactation (L) were analyzed on replicate Northern blots. Filters were hybridized with oligonucleotides specific for either the transgenic or wild-type (WT) WAP mRNAs.

**FIG. 3. Developmental patterns of WAP transgene expression in five lines of mice.** Levels of endogenous (dotted bars) and transgenic (solid bars) WAP RNAs during mammary development were quantitated by scintillation counting the respective bands from Northern blots (see Fig. 2). RNA samples from lines 3350 C and 3441 contained poly(A<sup>+</sup>)-enriched RNA equivalent to 100  $\mu$ g of total RNA. A direct comparison between the level of endogenous and allele-specific WAP mRNAs within one line was made possible by correcting for the different hybridization efficiencies of oligonucleotides using synthetic RNA standards (see "Materials and Methods"). A, line 3350A; B, line 3350B; C, line 3336; D, line 3350C; E, line 3441. Counts/min are shown on the ordinate, and the developmental time points are shown on the abscissa. P refers to the respective number of days of pregnancy and L to the number of days into the lactational period.

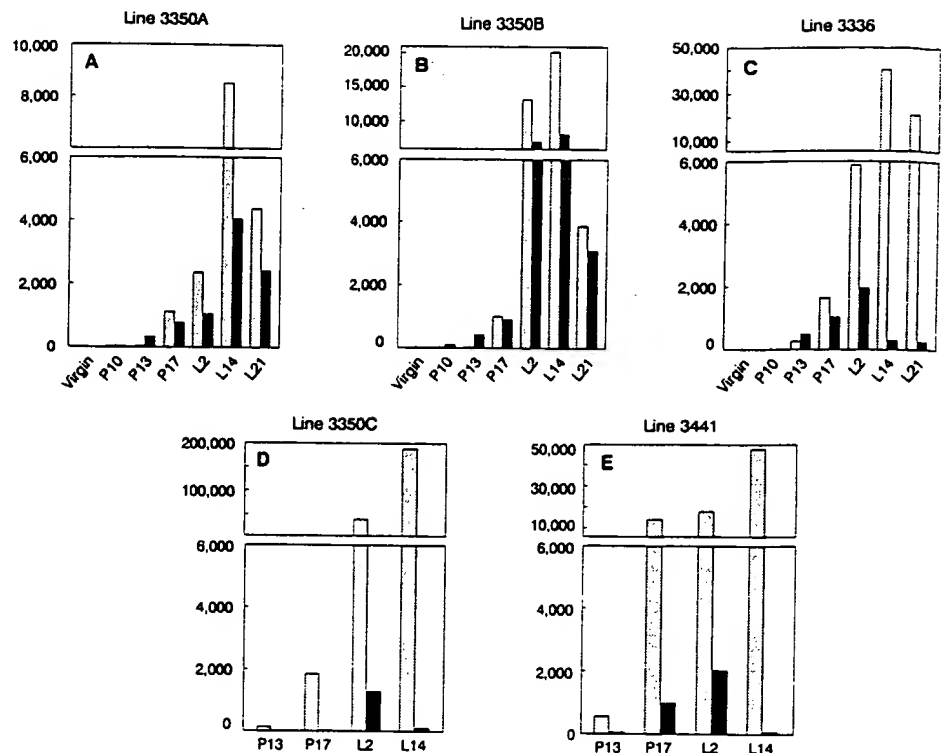


TABLE III

Expression of the WAP transgenes relative to the endogenous WAP gene during mammary development

The level of expression of the WAP transgenes at various stages during mammary gland development was quantitated on Northern blots and is presented as a percentage of the endogenous WAP gene at that stage.

Line	Allele	Expression of allele as percentage of endogenous WAP				
		P13	P17	L2	L14	L21
3350 A	KH	850	70	45	48	55
3350 B	KH	1080	90	54	40	80
3336	SSM	180	63	34	1	1
3441	BN	24	7	11	0.2	ND <sup>a</sup>
3350 C	KH	17	2	3	0.1	ND

<sup>a</sup> ND, not determined.

endogenous gene by at least 2 days. Furthermore, whereas the endogenous gene is induced about 1000-fold between day 13 of pregnancy and parturition, expression of the transgenes during this period in all lines, except 3350 C, increased less than 10-fold. Second, the activities of the WAP transgenes during lactation differed dramatically between lines of mice. Expression either continued to increase approximately to the same extent as the endogenous gene, as seen in lines 3350 A and B, or expression declined as in lines 3336, 3441 and 3350 C (Figs. 2 and 3). Presenting the concentration of transgenic WAP mRNA as a percentage of the endogenous mRNA further emphasizes the premature expression of transgenes, the similarities between the patterns of transgene expression during pregnancy, and differences during lactation (Table III).

The aberrant developmental expression of the WAP transgene alleles in different lines, especially during lactation, does not appear to be caused by the linker insertions or by differential expression of individual WAP genes within transgene loci. The three WAP gene alleles in the KSB lines 3336, 3441, and 3628 were integrated in a single locus, and expression during mammary development was coordinately regulated as

representatively shown for line 3336 (Fig. 2).

**In Vitro Hormonal Regulation of the WAP Transgenes in Mammary Tissue from Pregnant Mice**—To correlate the induction of the transgenes during development to hormonal regulation, we analyzed the expression of the WAP transgenes in an organ culture system. The WAP transgenes in lines 3350B and 3336 were already active in mammary tissue from mice which were 13–15 days pregnant (Fig. 4). In the presence of insulin, hydrocortisone, and prolactin, transgene expression in tissue from mice of lines 3350B and 3336 increased 4- and 2-fold, respectively (Fig. 4). Under the same conditions the endogenous WAP gene was induced over 30-fold. Expression of the WAP transgenes in tissue from mid-pregnant animals in the presence of insulin alone was below the level of detection, but was maintained in the presence of insulin and hydrocortisone and to a lesser extent with insulin and prolactin (Fig. 4). The levels of transgene mRNA detected in explants incubated with insulin and hydrocortisone were not due to retention of pre-existing mRNA but arose from continuing transcription of the transgenes, since comparable levels endogenous WAP mRNA were not maintained under these conditions in organ explants from late pregnant and lactating animals (Fig. 5 and data not shown).

Rehybridizations of RNA from explant cultures with a probe specific for the mouse  $\beta$ -casein mRNA demonstrated that, in common with the WAP transgenes, significant levels of  $\beta$ -casein mRNA were present at day 13 of pregnancy; induction in the presence of insulin, hydrocortisone, and prolactin (IFP) was only about 4-fold (Fig. 4). However, whereas expression of the WAP transgene appears to be more dependent on hydrocortisone than prolactin, the reverse is true for the  $\beta$ -casein gene (Fig. 4). Furthermore, the similarity between the  $\beta$ -casein blots from different explant experiments indicated that the response of the transgenes in different lines of mice can be compared directly.

**In Vitro Hormonal Regulation of the WAP Transgenes in Mammary Tissue from Lactating Mice**—The decline in expression of WAP transgenes during lactation in lines 3336,



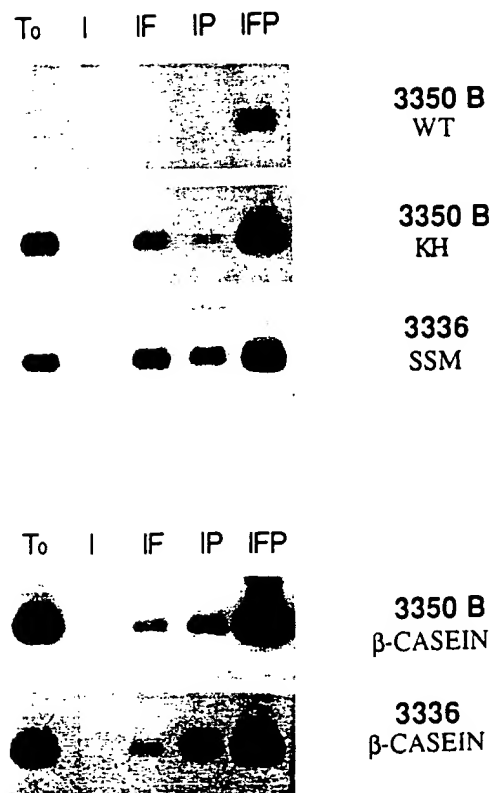


FIG. 4. Hormonal regulation of WAP transgenes in mammary tissue from mid-pregnant mice. Mammary explants were prepared from 13 to 15 day pregnant mice and RNA was extracted at the time of preparation ( $T_0$ ) or after 48 h in culture in the presence of insulin ( $I$ ), insulin and hydrocortisone ( $IF$ ), insulin and prolactin ( $IP$ ), or insulin, hydrocortisone, and prolactin ( $IFP$ ). Northern blots carrying 5  $\mu$ g of total RNA/lane were hybridized with oligonucleotides specific for either WAP alleles (upper panel) or mouse  $\beta$ -casein (lower panel).

3441, and 3350 C raised the question whether expression of the transgenes was generally repressed or had lost its response to a particular hormone. Tissue explants prepared from a fully lactating mouse from line 3336 were cultured under the conditions employed in the induction experiments shown in Fig. 4. Although the level of endogenous WAP RNA decreased at least 100-fold under any of the hormonal conditions, levels of the transgenic mRNA either increased marginally in the presence of insulin, hydrocortisone, and prolactin ( $IFP$ ) or remained the same with insulin and either hydrocortisone or prolactin (Fig. 5). Expression of keratin 18, a gene expressed in simple epithelial cells (18), increased during organ culture and was unaffected by hydrocortisone or prolactin. The overall response of the transgenes to hormones in tissue from lactating mice from line 3336 was similar to that obtained with tissue from pregnant mice. These results support the notion that the decrease in expression of the WAP transgenes during lactation in line 3336, and presumably also in lines 3441 and 3350 C, was due to a lack of sensitivity to the regulatory factors responsible for WAP gene induction during lactation. The transgenic WAP mRNA present during lactation in these lines probably reflects a basal level of transcription. A similar result was obtained *in vivo* with mammary tissue from mice of line 3336 where, after 3 days of weaning, levels of the endogenous WAP mRNA decreased about 100-fold and expression of transgenic WAP mRNA remained constant (data not shown).

#### DISCUSSION

Transgenic mice provide a unique tool for studying the developmental regulation of genes (19–21), especially those

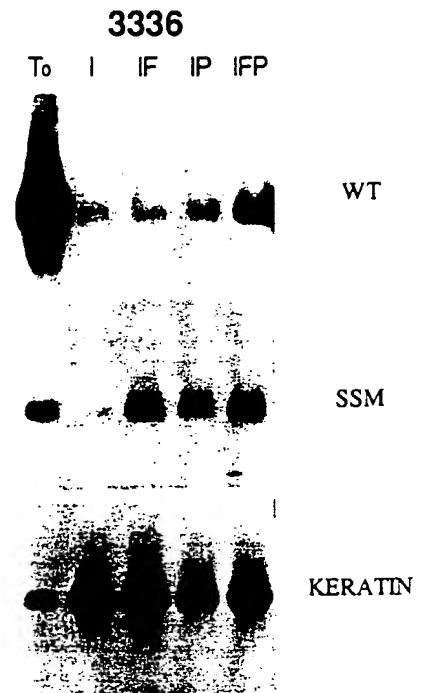


FIG. 5. Hormone-dependent expression of WAP transgenes in mammary tissue from lactating mice of line 3336. Mammary explants were prepared from a 10-day lactating 3336 mouse, and RNA was extracted at the time of preparation ( $T_0$ ) or after 48 h in culture in different combinations of insulin ( $I$ ), hydrocortisone ( $F$ ), and prolactin ( $P$ ). Northern blots carrying 5  $\mu$ g of total RNA/lane were hybridized with oligonucleotides specific for either WAP alleles or a DNA probe generated from a mouse keratin 18 cDNA (12).

such as the milk protein genes, which are only appreciably expressed in terminally differentiated cells (5, 22–24). We introduced a virtually unaltered 7.2-kb fragment, including the entire transcribed region of the mouse WAP gene, into mice and studied its developmental and hormonal regulation. The level of expression of the WAP transgene was at least an order of magnitude higher than several hybrid genes containing the WAP promoter (4, 5, 13, 25, 26). However, expression during mammary development and upon hormonal stimulation *in vitro* differed from the endogenous WAP gene. By comparing the developmental patterns of expression, and hormonal regulation of the WAP transgenes in six lines of mice, novel aspects of WAP gene regulation became apparent.

In contrast to the variation in overall patterns of transgene expression during mammary development, and especially during lactation (Fig. 3), the activity of WAP transgenes during mid-pregnancy in different lines of mice was similar. In all lines examined, expression of the WAP transgenes was detected at day 13 of pregnancy, thereby preceding the induction of the endogenous gene. Premature activation during pregnancy has also been observed with a hybrid gene containing the mouse WAP gene promoter (5) and a rat WAP transgene (27). Furthermore, between day 13 of pregnancy and parturition, a period in which the endogenous WAP gene was induced almost 1000-fold, expression of WAP transgenes in all lines except one increased less than 10-fold.

Results from organ culture experiments with mammary tissue from pregnant mice provided an insight into the basis of the temporal deregulation of the WAP transgene. The premature expression of WAP transgenes during pregnancy was presumably related to the high basal activity of the transgene; the activity was maintained in explant cultures with hydrocortisone and insulin, both of which are present throughout pregnancy. These observations suggest that the

transgene lacks a repressor element, or alternatively, the transgene array or surrounding chromatin results in the functional loss of a repressor. Consistent early expression and variation in both patterns of induction during pregnancy and response to hormones *in vitro* suggest that both situations may operate. Whereas the 30-fold induction of the endogenous WAP gene *in vitro* required the synergistic action of insulin, hydrocortisone, and prolactin, expression of the transgenes was only marginally induced by prolactin. The small *in vitro* induction of the WAP transgenes by prolactin correlates with the modest increase in expression prior to parturition and is presumably a consequence of the prematurely elevated expression levels in mid-pregnancy. In a manner similar to the response of a WAP-tPA transgene (5), prolactin was not necessary to maintain the level of transgenic WAP RNA in organ culture, but was required for continued expression of the endogenous WAP gene. Taken together the results are consistent with prolactin acting on the endogenous WAP gene through the release of repression, thereby facilitating access of the gene to regulatory factors dependent on insulin and hydrocortisone. In support of this model, results obtained with a cell culture system suggest that the mouse  $\beta$ -casein promoter is induced by hormones through the release of transcriptional repression.<sup>2</sup>

In contrast to the situation in pregnancy, expression of the WAP transgenes in individual lines of mice differed dramatically during lactation. In two lines expression of the WAP transgene approximately followed the pattern of the endogenous WAP gene. However, in three other lines of mice, in which the transgenes were induced during pregnancy and responded to hormones in organ culture, expression decreased in response to lactation. To obtain the same effect in three independent lines of mice demonstrates that loss of induction was not peculiar to one site of integration, but that the transgene lacks a regulatory element necessary to ensure correct expression during lactation. Günzburg and co-workers (28) have also reported the down-regulation of a WAP-hGH transgene in transgenic mice. Although a decrease in the expression of a 4.3-kb rat WAP transgene during lactation was not reported (27), this phenomenon may not have been detected, because developmental studies were performed on only two lines of mice and at a single time point during lactation. Alternatively, the rat and mouse WAP genes may have a different arrangement of regulatory elements.

Our results indicate that high level WAP gene expression during lactation is mediated by a combination of elements or mechanisms distinct from those operating during pregnancy. This is supported by the inability to maintain high levels of endogenous WAP RNA in tissue from lactating mice, under conditions sufficient to induce expression in tissue from pregnant mice. Expression of keratin 18 mRNA in culture (Fig. 5.) demonstrated that the decrease in endogenous WAP mRNA *in vitro* was not due to a general decrease in gene expression. Appropriate induction of WAP transgenes in two lines of mice (3350 A, 3350 B) during lactation indicates that chromatin at the sites of integration may have provided a positive influence which enabled the transgene to be appropriately induced during lactation. A similar facilitating effect of flanking chromatin has been described in a deletion analy-

sis of the locus activating region of a human adenosine deaminase transgene.<sup>3</sup>

The coordinate developmental regulation and equivalent expression of different WAP transgene alleles within a single locus suggest that position effects are exerted on the transgene array as a whole and are not due to local effects within the transgene cluster. This supports the notion that position effects may be mediated by some general characteristic of the surrounding chromatin, probably due to regulatory elements at some distance from the transgene array (29), and are not due to a local enhancer which might be expected to polarize expression within the transgene locus. These results demonstrate the feasibility of analyzing the function of mutated genes, relative to an unaltered control gene, by cointegration of transgenes in transgenic mice.

**Acknowledgments**—We thank the members of the Laboratory of Biochemistry and Metabolism for their advice, Robert Oshima for a mouse keratin cDNA clone, and W. Jakoby for his most generous support.

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<sup>2</sup> W. Doppler, personal communication.

<sup>3</sup> B. Aronow, personal communication.

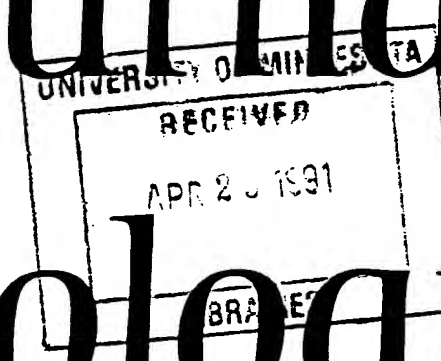
APRIL 15, 1991

VOLUME 266

NUMBER 11

ISSN 0021-9258  
JBCHA3 266(11) 6659-7312 (1991)

# THE Journal of Biological Chemistry

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# Biological Chemistry

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## CONTENTS\*

### MINIREVIEW

- 6659 **DNA topoisomerases: why so many?**  
*James C. Wang*

### COMMUNICATIONS

- 6663 **Deadenylation and turnover of interferon- $\beta$  mRNA.**  
*Karsten Peppel and Corrado Baglioni*
- 6667 **GC-rich DNA sequences block homologous recombination *in vitro*.**  
*Alexandra Gruss, Véronique Moretto, S. Dusko Ehrlich, Patrick Duwat, and Patrick Dabert*
- 6670 **Identification of a domain that mediates vesicle aggregation reveals functional diversity of annexin repeats.**  
*Joel D. Ernst, Elaine Hoyer, R. Alexander Blackwood, and Tina L. Mok*
- 6674 **Estradiol  $\beta$ -D-xyloside, an efficient primer for heparan sulfate biosynthesis.**  
*Fulgencius N. Lugemwa and Jeffrey D. Esko*
- 6678 **Phosphorylation of caldesmon by cdc2 kinase.**  
*Alan S. Mak, Mark H. Watson, Craig M. E. Litwin, and Jerry H. Wang*
- 6682 **Cloning of an alternate form of vascular cell adhesion molecule-1 (VCAM1).**  
*Catherine Hession, Richard Tizard, Cornelia Vassallo, Susan B. Schiffer, Deborah Goff, Pamela Moy, Gloria Chi-Rosso, Stephan Luhowskyj, Roy Lobb, and Laurelee Osborn*
- 6686 **Circumsporozoite protein gene from *Plasmodium reichenowi*, a chimpanzee malaria parasite evolutionarily related to the human malaria parasite *Plasmodium falciparum*.**  
*Altaj A. Lal and Ira F. Goldman*
- 6690 **<sup>31</sup>P-Phospho-NMR demonstration of phosphocysteine as a catalytic intermediate on the *Escherichia coli* phosphotransferase system *ETI*<sup>TM</sup>.**  
*Hendri H. Pas, Gert H. Meyer, Wim H. Kruizinga, Koos S. Tamminga, Rob P. van Weeghel, and George T. Robillard*

### ARTICLES

- 6693 **Evidence that the asparagine 322 mutant of the lactose permease transports protons and lactose with a normal stoichiometry and accumulates lactose against a concentration gradient.**  
*Peter J. Franco and Robert J. Brooker*
- 6700 **Role of protein phosphorylation in the maturation-induced activation of a myelin basic protein kinase from sea star oocytes.**  
*Jasbinder S. Sanghera, Harry B. Paddon, and Steven L. Pelech*
- 6708 **Evidence that the 90-kDa heat shock protein (HSP90) exists in cytosol in heteromeric complexes containing HSP70 and three other proteins with *M<sub>r</sub>* of 63,000, 56,000, and 50,000.**  
*Gary H. Perdew and Murray L. Whitelaw*
- 6714 **Structural determination and immunochemical characterization of the type V group B *Streptococcus* capsular polysaccharide.**  
*Michael R. Wessels, José L. DiFabio, Vicente-Javier Benedi, Dennis L. Kasper, Francis Michon, Jean-Robert Brisson, Jarmila Jelinková, and Harold J. Jennings*

- 6720 **Cholesterol esterase biosynthesis in rat pancreatic AR42J cells. Post-transcriptional activation by gastric hormones.**  
*Yan Huang and David Y. Hui*
- 6726 **The epithelium, endothelium, and stroma of the rabbit cornea generate (12*S*)-hydroxyeicosatetraenoic acid as the main lipoxygenase metabolite in response to injury.**  
*John S. Hurst, Michael Balazy, Haydee E. P. Bazan, and Nicolas G. Bazan*
- 6731 **Evidence that insulin plus ATP may induce a conformational change in the  $\beta$  subunit of the insulin receptor without inducing receptor autophosphorylation.**  
*Betty A. Maddux and Ira D. Goldfine*
- 6737 **Stabilization of thermally labile alkylcobalamins by a haptocorrin from chicken serum.**  
*Kenneth L. Brown, Harold B. Brooks, David Behnke, and Donald W. Jacobsen*
- 6742 **Deletion mutation in an eye lens  $\beta$ -crystallin. An animal model for inherited cataracts.**  
*Carolyn Chambers and Paul Russell*
- 6747 **Sequence specificities of human fibroblast and neutrophil collagenases.**  
*Sarah Netzel-Arnett, Gregory Fields, Henning Birkedal-Hansen, and Harold E. Van Wart*
- 6756 **Changes in the structure and biological property of *N*- $\rightarrow$ O sulfate-transferred, *N*-sulfated heparin.**  
*Hideki Uchiyama and Kinzo Nagasawa*
- 6761 **Cytokine-mediated regulation of ovarian function. Tumor necrosis factor  $\alpha$  inhibits gonadotropin-supported ovarian androgen biosynthesis.**  
*Cristiana L. Andreani, Donna W. Payne, Jeffrey N. Packman, Carol E. Resnick, Arye Hurwitz, and Eli Y. Adashi*
- 6767 **Isolation and characterization of a cDNA clone encoding an 18-kDa hydrophobic photosystem I subunit (PSI-L) from barley (*Hordeum vulgare* L.).**  
*Jens Sigurd Okkels, Henrik Vibe Scheller, Ib Svendsen, and Birger Lindberg Møller*
- 6774 **Interaction of C-terminal sequences of human immunodeficiency virus reverse transcriptase with template primer.**  
*Anthony L. DeVico, Terry D. Copeland, Stephen Oroszlan, Robert C. Gallo, and Mangalasseri G. Sarngadharan*
- 6780 **Transmembrane signal transduction and osmoregulation in *Escherichia coli*. Functional importance of the periplasmic domain of the membrane-located protein kinase, EnvZ.**  
*Shin-ichi Tokishita, Atsuko Kojima, Hirofumi Aiba, and Takeshi Mizuno*
- 6786 **Low molecular weight kininogen binds to platelets to modulate thrombin-induced platelet activation.**  
*Frank J. Meloni and Alvin H. Schmaier*
- 6795 **Stereochemical trends in copper amine oxidase reactions.**  
*Araba A. Coleman, Christine H. Scaman, Young J. Kang, and Monica M. Palcic*

\* The CONTENTS arranged by Subject Categories will be found immediately following these CONTENTS.

Full Instructions to Authors will be found in (1991) *J. Biol. Chem.* 266, 665-675, and reprints may be obtained from the editorial office.

9

## TRANSGENIC MICE WITH $\mu$ AND $\kappa$ GENES ENCODING ANTIPHOSPHORYLCHOLINE ANTIBODIES

BY URSULA STORB, CARL PINKERT,\* BENJAMIN ARP, PETER ENGLER,  
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Analysis of the antibody response on the cellular and molecular level is complicated by the fact that B lymphocytes are an enormously heterogeneous population with respect to the immunoglobulin genes they express. It has been possible to alleviate this obstacle by studying monoclonal populations of myeloma cells or by immortalizing individual B cells in hybridomas. However, these cells are generally arrested in a particular stage of differentiation and do not permit the study of the dynamics of cell development and interaction. The introduction of rearranged Ig genes into the germline of mice has been a method to study a monoclonal response on the level of the whole animal (1-3). Transgenic mice have provided a unique and powerful tool to analyze the expression of Ig genes. Transgenic mice are produced by microinjection of cloned genes into the male pronucleus of fertilized eggs, and implantation of the embryos into the uterus of a foster female (4). We have previously produced transgenic mice with the functional  $\kappa$  gene from the myeloma MOPC-21 (1). We found that the expression of this rearranged  $\kappa$  transgene is restricted to B lymphocytes (5, 6), and that coexistence in a B cell of transgenic  $\kappa$  and endogenous H chains prevents rearrangement of endogenous  $\kappa$  genes (7, 8). Apparently, allelic exclusion of  $\kappa$  genes is regulated by a feedback from a complete Ig molecule, not by free L chains. It was important to check these findings with another  $\kappa$  gene that contains a different V-region and 5' upstream sequences. Also, the MOPC-21  $\kappa$  chain previously used cannot be secreted alone. The possibility of feedback by  $\kappa$  chains that can be secreted on their own needs to be evaluated. Furthermore, it has been reported (3, 9) that heavy chain transgenes cause feedback inhibition of H gene rearrangement. It will be important to determine whether this finding can be generalized by using H transgenes with a different V region. Beyond simply establishing the fact that H genes, or their products, and H plus L chains cause feedback inhibition of H and  $\kappa$  gene rearrangement, the molecular mechanism will have to be addressed. It appears possible that insertion of the H chain into

This work was supported by grants CA/AI 25754, CA 35979, and HD 19488 from the National Institutes of Health (Bethesda, MD) to U. Storb; by NIH grant HD 17321 to R. L. Brinster; NIH training grants HD 07155 (to C. Pinkert) and GM 07270 (to P. Engler); by a postdoctoral fellowship to K. Gollahan, awarded by the Cancer Research Institute; and by a grant from the Genetic Systems Corporation to J. Manz.

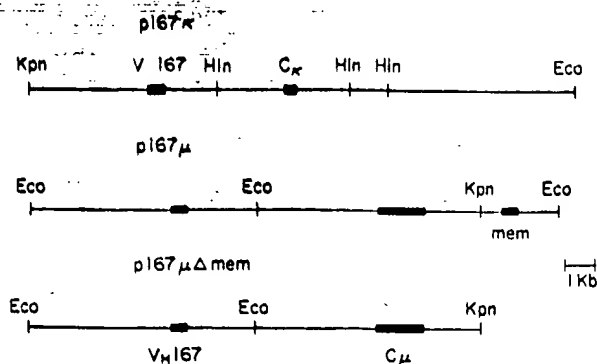


FIGURE 1. Maps of M167  $\kappa$  and  $\mu$  transgenes (see Materials and Methods for details). Kpn I, Hind III, and Eco RI restriction enzyme sites are indicated. Exons are shown by thick lines.

the surface or intracytoplasmic membranes of B cells is required for such feedback. We therefore wished to compare the expression of a complete  $\mu$  gene and of a stunted  $\mu$  gene lacking the membrane terminus ( $\mu \Delta$  mem), and compare the relative influence on endogenous H and L gene expression. The genes we chose to address these questions contain the V regions of the functional H and L genes of the myeloma MOPC-167. Transgenic mice were produced, which had introduced into their germline functionally rearranged  $V_{\kappa}$ -167- $C_{\kappa}$  genes and/or  $V_{H}$ -167- $C_{\mu}$  genes. The  $\mu$  gene is either complete or it lacks the portion that encodes the transmembrane sequence of  $\mu$  chains. The antibodies encoded by these genes react with phosphorylcholine (PC). The normal anti-PC response has been extensively studied by other laboratories (reviewed in 10), and these mice will be valuable in the analysis of the regulation of this response on the cellular and molecular level.

### Materials and Methods

**Enzymes.** Restriction endonucleases, ligases, etc. were obtained from New England Biolabs, Beverly, MA.

**Transgenes.** The transgenes are the functional  $\kappa$  gene of the myeloma MOPC-167, and  $\mu$  genes that were constructed from the V region of the functional  $\alpha$  H gene of the myeloma MOPC-167 and a germline  $C_{\mu}$  region (Fig. 1). The VJ and upstream region of the  $\kappa$  gene was a gift from P. Gearhart, The Johns Hopkins University, Baltimore, MD (11). It was joined at the Hind III site in the J- $C_{\kappa}$  intron to the 3' portion of the MOPC-21  $\kappa$  gene, which we had previously expressed in transgenic mice (1). The Eco-Eco 5' portions of the H genes were obtained from the MOPC-167  $\alpha$  gene (a gift of R. Perlmutter and L. Hood, California Institute of Technology, Pasadena, CA [12]) and ligated to the Eco-Eco 3' portion of a germline  $C_{\mu}$  gene (a gift of S. Cory and J. Adams, Walter and Eliza Hall Institute for Medical Research, Victoria, Australia). To produce the p167  $\mu \Delta$  mem gene, the  $\mu$  gene was cut at the Kpn I site. The  $\kappa$ ,  $\mu$ , and  $\mu \Delta$  mem genes were inserted into the plasmids pUC18, pUC13, and pUC19, respectively (13, 14). To produce transgenic mice, either the total plasmid was injected (linearized by cutting with Kpn I or Eco RI in case of the  $\kappa$  gene, or with Sal I in case of the  $\mu$  genes); or the inserts shown in Fig. 1 were excised with Eco RI and Pvu I in the case of  $\kappa$ , and Sal I and Pvu I in case of the  $\mu$  genes; this leaves ~30 bp from the pUC polylinkers and ~120 bp of the *lac* gene from pUC to help in the identification of positive mice. The restriction sites used to linearize or trim the plasmids, except for Kpn I, are located within the pUC vectors, and are not shown in Fig. 1.

**Transgenic Mice.** Transgenic mice were produced as described from (C57BL/6  $\times$

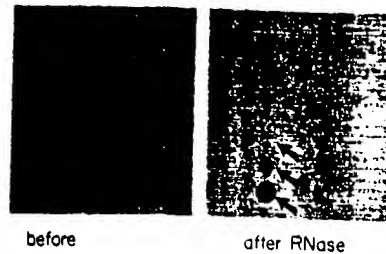


FIGURE 2. RNase treatment of RNA and DNA dots. Spleen and thymus RNAs (0.4 and 2  $\mu$ g) of normal and transgenic mice. The arrows point to dots of p167- $\kappa$  plasmid DNA. Hybridization with  $C_\mu$  probe. After exposure to x-ray film (left) the filter was treated with RNase and reexposed (right).

SJL)F<sub>2</sub> zygotes (4, 15). Positive mice were identified by dot hybridization of tail DNA with pUC DNA and V $\kappa$ -167 and/or V $\mu$ -167 probes, as previously described (1). The following groups of transgenic mice were produced:  $\kappa$  mice (contain only the 167- $\kappa$  gene);  $\mu$  mice (with the complete  $\mu$  gene);  $\mu$   $\Delta$  mem mice (with the membrane terminus-deleted  $\mu$  gene);  $\kappa$ ,  $\mu$ , and  $\kappa$   $\mu$   $\Delta$  mem mice (these are derived from ova coinjected with the  $\kappa$  and complete  $\mu$  genes or the  $\kappa$  and  $\mu$   $\Delta$  mem genes; thus, both genes are inserted at the same integration site);  $\kappa$   $\times$   $\mu$  mice (derived by breeding  $\kappa$  mice with  $\mu$  mice and selection of offspring positive for both genes; in these mice, the  $\kappa$  and  $\mu$  insertion sites are in different chromosomes). Offspring of the other groups were produced by mating of a transgenic mouse with a nontransgenic C57BL/6 or (C57BL/6  $\times$  5JL)F<sub>1</sub> hybrid mouse. We have not attempted to make homozygous mice with any of the transgenes.

**V $\kappa$ -167 and V $\mu$ -167  $C_\mu$  and  $C_\kappa$  probes.** The plasmid pSV $\kappa$ 167 contains a 310 bp Eco RI-Hinc II insert (the leader-V $\kappa$  region) isolated from the cDNA clone p167 $\kappa$  RI (16) and cloned into pSP65 (Promega Biotech, Madison, WI). The plasmid pSPV $\mu$ 167 contains a 189 bp Alu I, V $\mu$ -167-specific fragment subcloned from ChM167 $\alpha$ 10.1 (12) into pSP65. From these plasmids, 324- and 210-bp-long fragments, respectively, were excised by Eco RI-BamHI, which cut inside the polylinkers.

The  $C_\mu$  probe is an ~400 bp Pst I fragment isolated from pAB  $\mu$ -1, which is specific for  $C_\mu$  exons 3 and 4 (a gift of A. Bothwell, Yale University, New Haven, CT [17]).

The  $C_\kappa$  probe is a  $C_\kappa$  exon-specific fragment of ~500 bp isolated from pES201 (18).

The isolated fragments were ligated with T4 DNA ligase before nick translation.

**RNA-Dot Hybridization and Northern Blots.** RNA was prepared and Northern blots were performed with total organ RNAs as described (5). For dot hybridization, RNA was diluted in 15  $\times$  SSC and 10% formaldehyde, heated to 60°C for 15 min and applied to nitrocellulose filters prewashed in 15  $\times$  SSC using the Schleicher and Schuell (Keene, NH) minifold apparatus. Dots were washed with 15  $\times$  SSC. For DNA control dots on RNA dot filters, the DNAs were denatured in 0.1 M NaOH, 2 M NaCl by boiling for 1 min; for application to nitrocellulose, the denatured DNAs were diluted in 15  $\times$  SSC.

To assure that the RNA dot hybridizations were not due to contaminating DNA, all RNA preparations were extensively digested with RNase-free DNase I (Worthington Biochemical Corp., Freehold, NJ), and all RNA dots were also hybridized in parallel with vector DNA. In the case of  $\kappa$  transgenic mice, this generally did not give a signal. However, often with RNA from the  $\mu$  or  $\mu$   $\Delta$  mem mice carrying transgenes with vector attached, some hybridization was seen with vector DNA. This was not due to DNA contamination (see below), but apparently represented transcripts from vector DNA. Perhaps this is due to the fact that, in case of the  $\mu$  and  $\mu$   $\Delta$  mem genes, very little mouse DNA was present downstream of the poly(A) addition sites (in contrast to the  $\kappa$  gene); RNA polymerase seems to continue transcribing for a certain distance into the flanking vector sequences. The limits have not been defined. Finally, RNA dots were treated with RNase after hybridization. This eliminated RNA signals, but left DNA signals on the same filters intact (Fig. 2). For treatment with RNase, the dot filters were soaked in 0.2  $\times$  SSC for 5 min at 37°C. Then pancreatic RNase was added to 10  $\mu$ g/ml, and the incubation at 37°C was

continued for 30 min. The filters were extensively washed in 0.2× SSC, 0.1% SDS, 1 mM EDTA at 65°C before reexposure to x-ray film.

**DNA Quantitative Slot Hybridization.** DNA was denatured as above, diluted in 15× SSC, applied to nitrocellulose using a Schleicher and Schuell slot blotter, and hybridized with  $C_{\mu}$  or  $C_{\kappa}$  probes without vector in probe excess. Relative quantities of DNA were determined by scanning the slot hybridizations with a Helena Quick Scan (Helena Laboratories, Beaumont, TX). DNA from normal mice was included, and their  $C_{\mu}$  and  $C_{\kappa}$  signals represented two copies per genome.

**Transfection of DNA.** The  $\mu$  and  $\mu\Delta$  mem genes with their vectors were cotransfected together with the pSV2-gpt gene (19) into J558L myeloma cells (20) by electroporation (21).

**Immunofluorescence.** Thymuses were removed from the transgenic mice or normal littermate mice. A small piece was cut from the thymus for preparation of single-cell suspensions. The majority of the thymus was used for preparation of RNA. Each small thymus piece was gently rubbed over a 60-gauge stainless steel mesh. The cells were washed once in PBS plus 1% BSA (Sigma Chemical Corp., St. Louis, MO) and resuspended in PBS-BSA at a concentration of  $10^6$  cells/ml. The cells were then spun into ethanol-cleaned glass microscope slides  $10^5$  cells/slides, with a Shandon Southern Instruments, Inc. Cytospin (Sewickley, PA). The air-dried slides were fixed for 20 min at -20°C in 95% ethanol/5% acetic acid, then put through three 10-min washes of PBS. The antisera, tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse  $\mu$  (Cappel Laboratories, Cochranville, PA) and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse  $\kappa$  (Southern Biotechnology Associates Inc., Birmingham, AL), were diluted 1:200 in PBS-BSA and added directly to the fixed cells. The slides were incubated for 30 min at 37°C in a humidified chamber, then washed three times, once in PBS, once in PBS-BSA, and a final time in PBS. The cells were covered with PBS-glycerol and viewed with a Zeiss fluorescence microscope with transmitted light, a dark field condenser, and a 200 W high-pressure mercury bulb equipped with KP-546 and KP-500 excitation filters coupled to appropriate barriers to detect the fluorescence of rhodamine. The photographs were taken on Kodak Ektachrome daylight slide film, ASA 400.

## Results

**Transgene Copy Number.** The number of DNA molecules integrated in the transgenic mice varied between 1 and 87 (Table I). Most often the multiple copies were inserted at a single site, i.e., all positive offspring had the same copy number as the parent. In several cases, however, two integration sites were found that segregated in the offspring (indicated by A and B offspring in Table I; see also 1201 vs. 1202 and 1205 in Fig. 3). Transgenic mice that had been coinjected with  $\mu$  (or  $\mu\Delta$  mem) and  $\kappa$  genes had similar copy numbers of both genes integrated (one exception, 217-7 A, has only  $\mu$  and no  $\kappa$  genes) (Table I).

There was no good correlation between the transgene copy number and the level of RNA (Table I). This finding had also been made with MOPC-21  $\kappa$  transgenic mice (5). It is different from the relatively good correlation found in B cells transfected with Ig genes (22).

**Tissue-specific Expression of Transgenes.** We had previously (5) found that, in mice with a  $\kappa$  transgene from the myeloma MOPC-21, only B lymphocytes expressed the transgenic  $\kappa$  RNA. The question of tissue specificity was analyzed with many of the transgenic mice presented in this paper, examples of which are shown in Fig. 4. Liver, kidney, and heart do not contain transgenic  $\kappa$  or  $\mu$  RNAs beyond a low level expected from B cell contamination. Thus we have not found evidence for  $\mu$  expression in heart, as was found by Grosschedl et al. (2). Almost all the mice express the  $\mu$  and/or  $\kappa$  transgenes at a high level in spleen RNA



TABLE I  
DNA and RNA Quantitations of Some of the MOPC-167 Transgenic Mice

Transgene	Mouse number	Transgene copy number*			Spleen RNA†	
		$\kappa$	$\mu$	$\mu/\kappa$	$\kappa$	$\mu$
$\kappa$ vector $\Delta$	229-1	4			146	5
	230-3	1			47	3
	231-8	2			1 <sup>‡</sup>	3
	233-4	41			117	25
	233-8	13			58	5
	234-3	20			9	2
	234-4 A	11			233	1
	B	18				
$\kappa$	189-4	11			47	1
	194-2	2			47	1
$\mu$	199-9		70		<9	20
	200-3		3		50	60
	200-6		66		2	40
$\mu$ vector $\Delta$	243-2		1		2	3
	243-4		6		3	12
$\mu$ $\Delta$ mem	250-1		12		1	8
	250-2		2		1	8
	254-3		2		1	60
	254-3-11 <sup>§</sup>		2		1	60
	254-3-12 <sup>§</sup>		2		1	60
$\kappa\mu$	207-4	15	60	4	39	200
	210-3	3	4	1.3	20	30
	210-4	13	8	0.62	1.5	30
	212-2	10	19	1.9	3	25
	212-3	2	10	5	3	25
	212-5	37	23	0.62	10	100
$\kappa\mu$ $\Delta$ mem	216-1 A	1	2	2	12	100
	B	20	37	1.9		
	216-2	18	6	0.33	1	6
	216-7	1	2	2	12	20
	217-1	29	87	3	3	25
	217-4	8	17	2.1	6	25
	217-6	17	56	3.3	300	300
	217-7 A	0	2	>2	6	25
	B	4	24	6		

The mice shown contain the transgenes with the complete pUC vectors (see Fig. 1), except the  $\kappa$  vector del, the  $\mu$  vector del, and the  $\mu$   $\Delta$  mem groups, which contained only ~150 bp of the vector (see Materials and Methods).

\* Transgene copy number was determined by quantitative slot hybridization (see Fig. 3) on tail DNA of offspring from the original set of transgenic mice. A and B represent offspring with different copy numbers indicating that the parent had two independently segregating transgene insertion sites.

† Quantities of spleen 167 $\kappa$  or 167 $\mu$  RNA expressed as multiples of the levels in nontransgenic control mice. These determinations were made on the spleen RNA of the original transgenic mice, except in two offspring of mouse 254-3 (§).

‡ Because the presence of two adjacent, strongly hybridizing dots obscures the interpretation of the autoradiograph, the  $\kappa$  level for mouse 231-8 may actually be above normal.

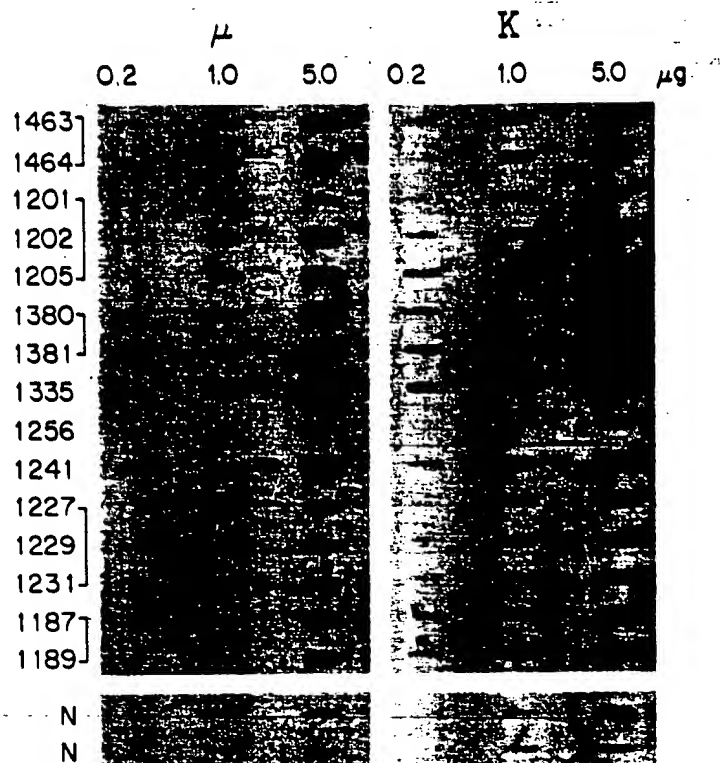


FIGURE 3. Quantitative slot hybridization of tail DNA samples. Shown are the DNAs from 15 offspring of  $\kappa\mu$  or  $\kappa\mu \Delta$  mem transgenic mice produced by coinjection of the 167- $\kappa$  and 167- $\mu$  or  $\mu \Delta$  mem genes, and of two nontransgenic (N) mice. Offspring from the same transgenic parent mated with a nontransgenic mouse are bracketed; from top to bottom the following transgenic lines are shown: 217-4, 216-1, 217-6, 217-1, 216-7, 207-4, 212-3, 212-2. The blots were hybridized with probes for  $C_\mu$  ( $\mu$ ) or  $C_\kappa$  ( $\kappa$ ), as indicated.

(Table I). In nontransgenic littermates, no or very low amounts of the  $V_\kappa$ -167- and  $V_H$ -167-positive RNAs are found in the spleen (N in Figs. 4, 5, and 7). In the thymuses of mice carrying  $\mu$ ,  $\kappa\mu$ ,  $\kappa \times \mu$ , or  $\kappa\mu \Delta$  mem transgenes,  $\mu$  RNA with  $V_H$ -167 sequence is found at about one-fifth to one-half the level of  $V_H$ -167 RNA in the spleen (Figs. 4 and 5, and data not shown). This confirms, with a different  $V_H$  gene, the finding of Grosschedl et al. (2) that rearranged  $\mu$  transgenes are expressed in T cells. Interestingly, in mice that carry both  $\mu$  and  $\kappa$  transgenes, only  $\mu$ , and not  $\kappa$  transcripts are seen in thymus (Fig. 4,  $\kappa \times \mu$ , and data not shown).

$\kappa$  transgenes are generally not expressed in the thymus, both in the case of the mice carrying the MOPC-21  $\kappa$  transgene previously analyzed (5), and in the  $\kappa$ -167 mice reported here. However, the two  $\kappa$  mouse lines shown in Fig. 4 consistently had significant quantities of  $\kappa$  transgenic RNA in the thymus. Reduction of plasma cells to  $\sim 0.4\%$  from thymus cell preparations by anti-Ia serum and complement did not eliminate the  $\kappa$  RNA (data not shown). No  $\kappa$  protein could be detected by immunofluorescence (not shown). These mice had a 58- and 117-fold increases of  $\kappa$ -167 RNA in spleen, compared with normal mice (Table I, 233-8 and 233-4). It appears possible that the  $\kappa$  RNA in the thymus is entirely due to B cells. However, we have not ruled out the possibility

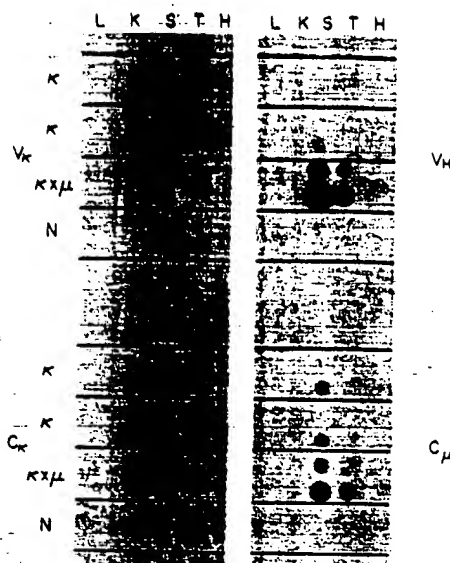


FIGURE 4. RNA dots of mouse organs. Each RNA was dotted as 2 and 10  $\mu$ g for the  $V_{\kappa}$  and  $V_{\mu}$  hybridizations, and 0.5 and 2.5  $\mu$ g for the  $C_{\kappa}$  and  $C_{\mu}$  hybridizations. L, liver; K, kidney; S, spleen; T, thymus; H, heart. On the left side, from top to bottom, the mice are indicated:  $\kappa$ : 233-8-3 and 233-4-2 are offspring of transgenic mice with the  $\kappa$ -167 gene without vector;  $\kappa \times \mu$ : an offspring from a mating between  $\kappa$  transgenic mouse 189-4 and  $\mu$  transgenic mouse 199-9, the complete vector is present in these transgenes; N: nontransgenic littermate of the  $\kappa \times \mu$  mouse.  $V_{\kappa}$ ,  $C_{\kappa}$ ,  $V_{\mu}$ , and  $C_{\mu}$  indicate the hybridization probes.

that the  $\kappa$  transgenes are actually expressed in T cells of these mouse lines, perhaps as a result of the relatively high copy number (13 and 41 copies) or chromosomal position. In the case of T cells transfected in vitro with  $\kappa$  genes, 5% of the transfectants expressed  $\kappa$  (22).

**$\kappa$  and  $\mu$  RNAs in Spleens of  $\kappa$  and/or  $\mu$  Transgenic Mice.** In mice that carry both the 167- $\kappa$  and  $\mu$  or  $\mu \Delta$  mem transgenes, there is generally a good correlation between the relative levels of H and  $\kappa$  RNAs with the V167-H or  $\kappa$  sequences (Fig. 5). In most cases, the levels of transgenic H RNA are somewhat higher than those of transgenic  $\kappa$  RNA. This is not an artifact of the specific activities of the hybridization probes; in fact, the signal with the  $V_{\kappa}$ -167 probe is higher than the  $V_{\mu}$ -167 signal when the probes are hybridized with equal amounts of the respective plasmid DNAs on the same blot (Fig. 5, *bottom*).

In two of the mice, the 167- $\kappa$  RNA levels were almost as low as in normal mice (fourth and sixth columns in the  $\kappa \mu$  and  $\kappa \mu \Delta$  mem mice of Fig. 5, respectively). Both these mice had relatively high copy numbers (13 and 18 copies) of the  $\kappa$  transgenes. We have no explanation for the minimal  $\kappa$  transgene expression. Both mice produced normal levels of total  $\kappa$  RNA in the spleen (not shown).

In general, there is a wide variation in the expression of  $\kappa$  and/or  $\mu$  RNA between individual mice. This does not seem to be related to the gene copy number (Table I). If this variability were dependent upon the insertion site of the transgenes, offspring of high expressors should also be high expressors, and vice versa with low expressors. In one particular strain of MOPC-167  $\kappa$ -transgenic mice, we found high expression over two generations, but low expression in the second offspring generation (R. L. O'Brien and U. Storb, unpublished data).

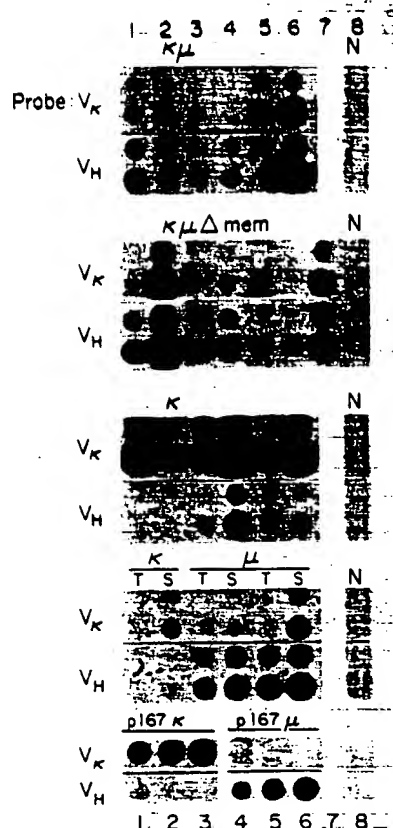


FIGURE 5. RNA dots of spleen and thymus. In each row the top dot is 5  $\mu$ g, the bottom dot 25  $\mu$ g of total RNA. Each group of transgenic mice is indicated on the top of the panels:  $\kappa\mu$ , eggs coinjected with the  $\kappa$ -167 and  $\mu$ -167 genes with the vector (212-5, 212-3, 212-2, 210-4, 210-3, and 207-4);  $\kappa\mu \Delta$  mem, eggs coinjected with the  $\kappa$ -167 and  $\mu$ -167  $\Delta$  mem genes with the vector (217-7, 217-6, 217-4, 217-1, 216-7, 216-2, and 216-1);  $\kappa$ , eggs injected with the  $\kappa$ -167 gene without the vector (third panel) (234-4, 234-3, 233-8, 233-4, 231-8, and 229-1) or with the vector (fourth panel) (189-4);  $\mu$ , eggs injected with the  $\mu$  167 gene with the vector (200-6 and 200-3). *N*, spleen of normal littermates of mice in the same panel, except in the fourth panel where *N* is (SJL  $\times$  C57BL/6) $F_1$  spleen. The three top panels show spleen RNA, the fourth panel shows thymus (*T*) and spleen (*S*) RNAs. The probes were  $V_{\kappa}$ -167 or  $V_{\mu}$ -167, as indicated. The probes were excised from the vector and contain essentially no vector DNA, as shown on DNA dots (bottom): p167- $\kappa$  and p167- $\mu$  are dots of the plasmid DNAs, including the vector.

Thus, perhaps the environmental levels of PC vary in our mouse colony (see Discussion). The variability in RNA levels will be further investigated.

**Immunoglobulin Protein in T Cells.** Thymocytes from a mouse carrying the 167- $\mu$  and 167- $\kappa$  transgenes were analyzed for  $\mu$  and  $\kappa$  proteins by immunofluorescence (Fig. 6). ~60% of the transgenic thymocytes contained  $\mu$  (Fig. 6D). In normal thymus, only ~0.1% of the cells were  $\mu^+$  (Fig. 6B); these appear to have plasma cell morphology. When stained with anti- $\kappa$ , only a few plasma cells, but not the thymocytes of the  $\kappa\mu$ -transgenic mouse were stained (Fig. 6F). The  $\mu^+$  cells are indeed T lymphocytes, because 98% of the thymocytes were Thy-1 $^+$  (not shown). Thymocytes from the same  $\kappa\mu$  mouse were also stained for surface immunofluorescence with anti- $\mu$ , and analyzed by FACS (not shown). The thymocytes were found to be negative for surface-bound immunoglobulin.

This particular  $\kappa\mu$  mouse (207-4) had very high levels of  $\kappa$  and  $\mu$  RNAs in the

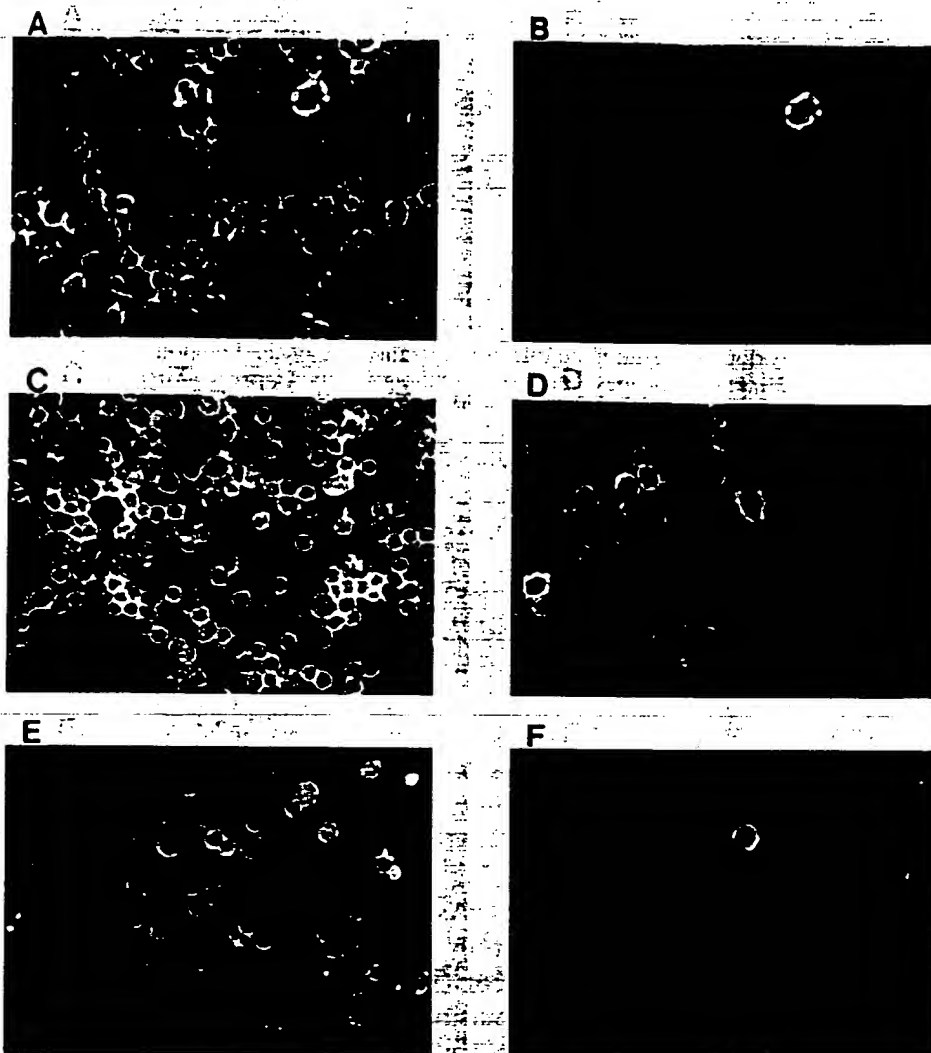


FIGURE 6. Immunofluorescence of thymocytes. Thymocytes of normal (A and B), and  $\kappa\mu$  transgenic mice (C-F) (207-4) were stained with rhodamine-conjugated anti- $\mu$  (A-D) or anti- $\kappa$  (E and F) and photographed in darkfield (A, C, and E) or fluorescence microscopy (B, D, and F).

spleen (Fig. 5,  $\kappa\mu$  column 6), and of  $\mu$  RNA in the thymus (not shown). The thymuses of two other  $\kappa\mu$  mice (212-2 and 212-3) were also analyzed for cytoplasmic immunofluorescence and found to be weakly positive for  $\mu$  protein in a high proportion of the thymocytes (not shown).

*Influence of Transgenes on Expression of Endogenous MOPC-167-like Genes.* Several of the mice that carry only  $\kappa$ -167 transgenes show, in addition to a large amount of  $V_{\kappa}$ -167 RNA, also a several-fold increased level of H RNA with the  $V_H$ -167 sequence compared with normal littermates (Fig. 5,  $\kappa$ ; Table I,  $\kappa$  vector  $\Delta$ ). This RNA is encoded by endogenous H genes. There may be some correlation between the amount of transgenic  $\kappa$ -167 and endogenous H-167 RNA, since only the mice with very high levels of  $\kappa$ -167 RNA show an increase of H-167 RNA. However, some mice with very high levels of  $\kappa$ -167 do not have high levels

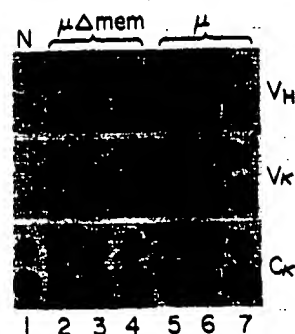


FIGURE 7. RNA dots of spleen from different  $\mu$  transgenic mice. The dots contain 2 and 10  $\mu$ g RNA for the  $V_H$  and  $V_\kappa$  probes, and 0.5 and 2.5  $\mu$ g for the  $C_\kappa$  probe. Mice from which the spleen RNAs were obtained are indicated on the top.  $N$ , nontransgenic littermate of the  $\mu\Delta$  mem mice; 2-4 are 250-1, 250-2, and 254-3; 5-7 are 243-4, 243-2, and 200-3. The hybridization DNA probes are indicated on the right side.

of H-167 RNA (Fig. 4  $\kappa$  mice; Fig. 5, third panel,  $\kappa$ , column 1; Table I mouse 234-4).

Likewise, mice with the complete 167- $\mu$  gene show relatively high levels of 167- $\kappa$  RNA in the spleen (Figs. 5 and 7,  $\mu$ ). Of five total mice with the 167- $\mu$  transgene, all had increased expression of endogenous 167- $\kappa$ -like genes. In contrast, no mice ( $n = 5$ ) with the 167  $\mu \Delta$  mem gene showed an increased amount of 167- $\kappa$  RNA (Fig. 7 shows three of these; see also Table I), despite high levels of 167-H RNA and total  $\kappa$  RNA (probed with  $C_\kappa$ ). These results are significant with respect to B cell triggering (see Discussion).

**Types of 167 Heavy Chain RNAs.** Northern blots of spleen RNA probed with  $V_H$ -167 confirmed that normal mice (Fig. 8,  $N$ ) do not produce detectable levels of  $V_H$ -167-containing RNA (Fig. 8A). As controls, the two  $\mu$  genes were transfected into the myeloma J558L, and only the secreted form of the 167- $\mu$  mRNA was seen, even with the complete  $\mu$  gene (Fig. 8C). This reflects RNA processing in favor of the secreted form in the plasma cell stage. However, transgenic mice with the complete  $\mu$  gene show both the membrane and the secreted form of 167- $\mu$  RNA in spleen and thymus (Fig. 8, A and C). In the spleen, the secreted form is predominant, probably indicating that the majority of the 167- $\mu$  RNA is derived from plasma cells. In the thymus, an equal or greater amount of the  $V_H$ -167 RNA is in the membrane form. Thus, T cells appear to process  $\mu$  RNA like pre-B and early B lymphocytes, with both polyadenylation sites of  $\mu$  being used. We do not know how these steady-state levels of  $\mu$  RNA in the transgenic thymus are influenced by differential stabilities versus transcription rates. Besides the defined bands of secreted and membrane 167- $\mu$  RNA (Fig. 8, A and C) two shorter-length transcripts are seen when Northern blots of thymus RNA are probed with  $C_\mu$  (Fig. 8B). These incomplete transcripts do not contain  $V_H$ -167 sequences (compare, in Fig. 8, A with B). They are very prominent in the thymus of mice with or without  $\mu$  transgenes (Fig. 8 B and other data not shown). In spleens of mice with or without a  $\mu$  transgene, only the incomplete  $\mu$  RNA of the higher molecular mass is observed (Fig. 8 B, and not shown). These short transcripts in spleen and thymus probably represent RNA transcripts from DJ rearrangements of endogenous H genes (23, 24).

Mice with the  $\mu \Delta$  mem transgene have only the secreted form of the  $V_H$ -167-

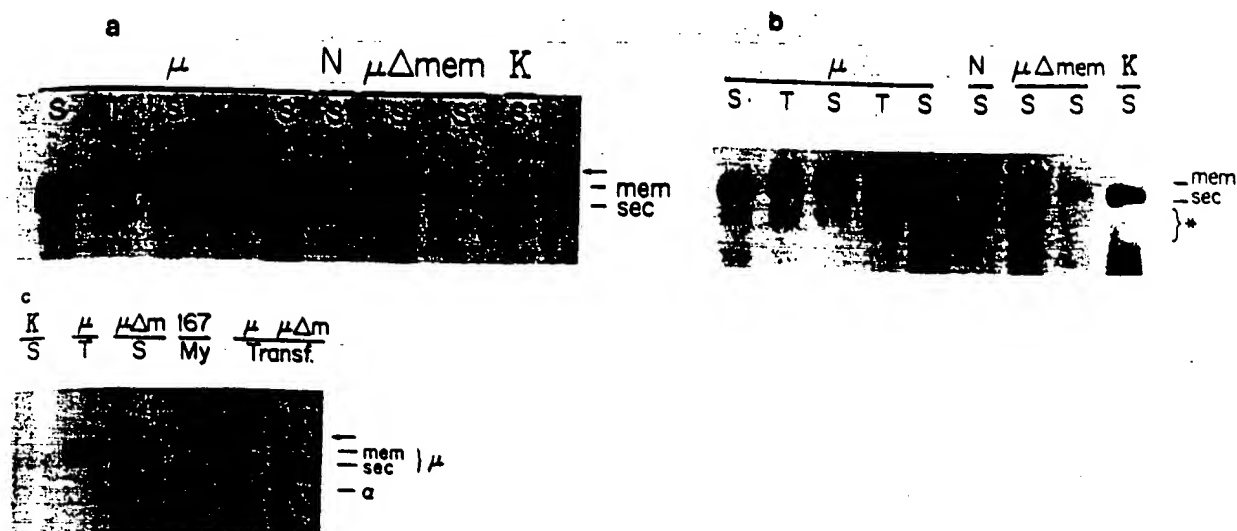


FIGURE 8. Northern blots. Spleen (S) and thymus (T) RNAs from transgenic and normal (N) mice; the transgenic mice contained the p167- $\mu$  gene ( $\mu$ ) (200-3, 200-6, and 207-4), the p167- $\mu$   $\Delta$  mem gene ( $\mu\Delta$  mem) (217-6), or the p167- $\kappa$ (K) gene (233-4); RNA from the myeloma MOPC 167 (167/my); and RNAs from the myeloma J 558L transfected with the p167 $\mu$  and p167 $\mu$  mem gene ( $\mu$ ,  $\mu\Delta$  mem/Transf.). A and B are the same blots (20  $\mu$ g RNA each lane) hybridized in A with the V<sub>H</sub>-167 probe, and after decay of the signal, rehybridized with C <sub>$\mu$</sub> . C, 30  $\mu$ g RNA for S and T, 10  $\mu$ g for the rest, hybridized with V<sub>H</sub>-167. Mem and sec: membrane (2.7 kb) and secreted (2.4 kb) forms of  $\mu$  mRNA.  $\alpha$ , marker for 1.9 kb size of secreted form of  $\alpha$  and  $\gamma$  mRNAs. Arrow indicates high-mol-wt form in spleen RNA of two different p167  $\mu\Delta$  mem transgenic mice. Asterisk indicates incomplete  $\mu$  transcripts.

$\mu$  RNA in their spleen, as expected (Fig. 8, A and C). In two cases, the spleen RNAs contain a large transcript of  $\sim 3$  kb in addition to the secreted form (Fig. 8, A and C). This is  $\sim 300$  nucleotides larger than the membrane form. Interestingly, this transcript does not contain C <sub>$\mu$</sub>  sequences (Fig. 8B). We have not yet investigated the origin of the long transcripts. Perhaps transcription into flanking sequences and aberrant splicing is involved.

The endogenous 167- $\mu$  RNA produced in the spleen of mice with the 167- $\kappa$  transgene did not contain any detectable membrane form (Fig. 8, A and C  $\kappa$ /S). In addition to the secreted  $\mu$  form, one of the  $\kappa$  mice also contained a detectable amount of V<sub>H</sub>-167<sup>+</sup> RNA of the size of secreted  $\gamma$  or  $\alpha$  RNA (Fig. 8C).

### Discussion

The MOPC-167  $\mu$  and  $\kappa$  genes are expressed at high levels in the transgenic mice reported here. This study represents the first attempt at expressing a  $\mu$  gene with deleted membrane terminus, either in cell transfection or when propagated through the germline of mice. It clearly shows that the region just 5', within, and 3' of the  $\mu$  membrane terminus is not required for correct expression and processing of secreted  $\mu$ . This is apparently in contrast to the C <sub>$\kappa$</sub>  gene, where we have preliminary evidence that a region 6 kb 3' of C <sub>$\kappa$</sub>  is required for high expression in transgenic mice (U. Storb, C. Pinkert, R. Brinster, and S. L. McKnight, unpublished).

The  $\mu$  and  $\kappa$  transgenes are generally transcribed in a tissue-specific fashion. However, the  $\mu$  transgenes are expressed in thymus, where normally correctly rearranged  $\mu$  genes do not exist. Thus, T cells seem to have the capacity to

activate  $\mu$  genes sufficiently for transcription, but they seem to lack the ability to correctly rearrange Ig genes. The V<sub>H</sub> 167-containing transcripts in the transgenic thymuses are of normal length for secreted and membrane  $\mu$ -encoding mRNAs. The mRNAs are apparently stable and translated into stable  $\mu$  protein, which can be visualized in thymocyte cytoplasm. The  $\mu$  protein cannot be detected on the cell surface by immunofluorescence, and one may conclude that  $\mu$  does not associate with the  $\alpha$  or  $\beta$  chain of the T cell receptor, or at least not in a way that allows binding of anti-C $\mu$  antibodies. Only ~60% of the thymocytes showed intracytoplasmic  $\mu$  protein (Fig. 6D). In normal thymus, ~50–70% of the thymocytes have T cell receptor protein on the surface (25). It is reasonable to postulate that these same cells would express  $\mu$  genes, although we have not made the direct correlation. Presumably the same *trans* acting factors for gene expression that interact with T cell receptor genes can also activate  $\mu$  genes.

Most of the transgenic mice express the  $\mu$  and  $\kappa$  genes at a high rate in the spleen. Furthermore, most of the transgenic mice produce 10–100-fold higher levels of anti-PC antibodies than normal mice (C. Pinkert, J. Manz, R. L. Brinster, and U. Storb, unpublished observations). For B cell activation to antibody secretion, antigen is probably required. Phosphorylcholine, the target antigen for MOPC-167 antibodies, is a fairly ubiquitous antigen, as it is a component of bacterial membranes, fungi, parasites, etc. (26, 27). The levels of anti-PC antibodies and of endogenous 167- $\kappa$  RNA are greatly increased in transgenic mice with the complete  $\mu$  gene (and conversely, endogenous 167- $\mu$  RNA in  $\kappa$  mice). Apparently, B cells that express the 167- $\mu$  gene and at the same time happen to express an endogenous 167- $\kappa$  transgene are triggered and expanded at a much higher rate than B cells that express other endogenous  $\kappa$  genes.

Mice with the  $\mu$   $\Delta$  mem transgene produce high levels of mRNA encoded by this gene. In contrast to the mice with the complete  $\mu$  gene, however, they do not produce an increased amount of MOPC-167-like  $\kappa$  RNA. This difference probably indicates that B cells expressing the  $\mu$   $\Delta$  mem transgene are not activated by PC. In contrast to membrane Ig, the secreted molecule lacks a hydrophobic carboxy terminus required for stable anchorage in the membrane. Recent work (28, 29) has shown that the role of membrane Ig in B cells is to capture antigen for endocytosis and processing. Processed antigen is then presented to T cells in association with Ia, not in association with surface Ig. Dependent on the carrier, PC can be a T-dependent antigen (30). It appears that, in transgenic mice with the  $\mu$   $\Delta$  mem gene, B cells that produce the transgenic secreted H chain together with endogenous M-167  $\kappa$  chains are not selected. Although secreted Ig molecules are released from the cell surface, they apparently do not dwell in the plasma membrane in a way that would permit cell activation. It is curious, then, that mice with the  $\mu$   $\Delta$  mem transgene produce such high levels of the secreted M. 167  $\mu$  RNA. The high transgenic and total endogenous  $\mu$  mRNA levels in these mice (Fig. 8, A and B) suggest that most B cells express the  $\mu$   $\Delta$  mem transgenes together with complete endogenous membrane immunoglobulins. This would mean that the  $\mu$   $\Delta$  mem gene or its encoded secretory  $\mu$  protein does not cause feedback inhibition of endogenous H and L gene rearrangement. As the transgenic  $\mu$   $\Delta$  mem B cells with endogenous H and L chains become activated by any



of a large number of antigens, the transgenic mRNA probably becomes amplified together with the endogenous Ig mRNAs in the same cell.

While the  $\mu$   $\Delta$  mem protein molecules would not interfere with efficient triggering of B cells that express endogenous Igs, they do apparently associate with endogenous L chains in secreted molecules (not shown). Thus, ~50% scrambled (transgenic  $\mu$   $\Delta$  mem H plus endogenous H and L) antibody molecules and 25% pure  $\mu$   $\Delta$  mem H, endogenous L molecules (in addition to 25% pure endogenous HL molecules), would be secreted by most individual plasma cells. Nevertheless, the mice, now ~1 yr old, appear to be healthy without special protection from pathogens. Apparently, the multilevel immune defense system contains enough safeguards that dilution of pure antibodies by a high proportion of scrambled and irrelevant molecules can be tolerated. With respect to this plasticity of the immune system, and in relation to allelic exclusion, it will be interesting to determine on the cellular level how the coexpression of endogenous Ig genes and transgenes is controlled.

### Summary

Transgenic mice were produced that carried in their germlines rearranged  $\kappa$  and/or  $\mu$  genes with  $V_{\kappa}$  and  $V_H$  regions from the myeloma MOPC-167  $\kappa$  and H genes, which encode anti-PC antibody. The  $\mu$  genes contain either a complete gene, including the membrane terminus ( $\mu$  genes), or genes in which this terminus is deleted and only the secreted terminus remains ( $\mu$   $\Delta$  mem genes). The  $\mu$  gene without membrane terminus is expressed at as high a level as the  $\mu$  gene with the complete 3' end, suggesting that this terminus is not required for chromatin activation of the  $\mu$  locus or for stability of the mRNA. The transgenes are expressed only in lymphoid organs. In contrast to our previous studies with MOPC-21  $\kappa$  transgenic mice, the  $\mu$  transgene is transcribed in T lymphocytes as well as B lymphocytes. Thymocytes from  $\mu$  and  $\kappa\mu$  transgenic mice display elevated levels of M-167  $\mu$  RNA and do not show elevated levels of  $\kappa$  RNA, even though higher than normal levels of M-167  $\kappa$  RNA are detected in the spleen of these mice. ~60% of thymocytes of  $\mu$  transgenic mice produce cytoplasmic  $\mu$  protein. However, despite a large amount of  $\mu$  RNA of the membrane form,  $\mu$  protein cannot be detected on the surface of T cells, perhaps because it cannot associate with T cell receptor  $\alpha$  or  $\beta$  chains.

Mice with the complete  $\mu$  transgene produce not only the  $\mu$  transgenic mRNA but also considerably increased amounts of  $\kappa$  RNA encoded by endogenous MOPC-167 like  $\kappa$  genes. This suggests that B cells are selected by antigen (PC) if they coexpress the  $\mu$  transgene and appropriate anti-PC endogenous  $\kappa$  genes. Mice with the  $\mu$   $\Delta$  mem gene, however, do not express detectable levels of the endogenous MOPC-167  $\kappa$  mRNA. Like the complete  $\mu$  transgene, the M-167 $\kappa$  transgene also causes amplification of endogenous MOPC-167 related immunoglobulins; mice with the  $\kappa$  transgene have increased amounts of endogenous MOPC-167-like  $\mu$  or  $\alpha$  or  $\gamma$  in the spleen, all of the secreted form. Implications for the regulation of immunoglobulin gene expression and B cell triggering are discussed.

We thank Drs. P. Gearhart, L. Hood, R. Perlmutter, S. Cory, J. Adams, A. Bothwell, J.

Mulligan, and P. Berg for cloned genes; S. Morrison for myeloma J558L; J. Hagman for RNA from transfected cells; M. Trumbauer and M. Avarbock for technical assistance; and J. Clagett for use of the cytocentrifuge and fluorescent microscope.

Received for publication 10 February 1986 and in revised form 12 May 1986.

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VOLUME 164

1986

PUBLISHED MONTHLY BY  
THE ROCKEFELLER UNIVERSITY PRESS



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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>C12N 15/85, 15/57</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 90/05188</b> <b>(43) International Publication Date:</b> 17 May 1990 (17.05.90)
<b>(21) International Application Number:</b> PCT/GB89/01343 <b>(22) International Filing Date:</b> 13 November 1989 (13.11.89) <b>(30) Priority data:</b> 8826446.0 11 November 1988 (11.11.88) GB <b>(71) Applicant (for all designated States except US):</b> PHARMACEUTICAL PROTEINS LIMITED [GB/GB]; Orchard Brae House, 30 Queensferry Road, Edinburgh EH4 2HG (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> ARCHIBALD, Alan, Langskill [GB/GB]; 70 Silverknowes View, Edinburgh EH4 5PS (GB). CLARK, Anthony, John [GB/GB]; 29 Broomieknowe, Lasswade, Midlothian EH18 1LN (GB). HARRIS, Stephen [GB/GB]; 3 Leamington Road, Edinburgh EH3 9PD (GB). McCLENAGHAN, Margaret [GB/GB]; 8 Livingstone Place, Edinburgh EH9 1PA (GB). SIMONS, John, Paul [GB/GB]; 94 Warrender Park Road, Edinburgh EH9 1ET (GB). WHITE LAW, Christopher, Bruce, Alexander [GB/GB];		43 Viewforth, Edinburgh EH10 4LA (GB). <b>(74) Agents:</b> SHEARD, Andrew, Gregory et al.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB). <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), SU, US. <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> A GENETIC CONSTRUCT OF WHICH PROTEIN-CODING DNA COMPRISES INTRONS AND IS DESIGNED FOR PROTEIN PRODUCTION IN TRANSGENIC ANIMALS		
<b>(57) Abstract</b> <p>Proteinaceous products can be produced by transgenic animals having genetic constructs integrated into their genome. The construct comprises a 5'-flanking sequence from a mammalian milk protein gene (such as beta-lactoglobulin) and DNA coding for a heterologous protein other than the milk protein (for example a serin protease such as alpha<sub>1</sub>-antitrypsin or a blood factor such as Factor VIII or IX). The protein-coding DNA comprises at least one, but not all, of the introns naturally occurring in a gene coding for the heterologous protein. The 5'-flanking sequence is sufficient to drive expression of the heterologous protein.</p>		

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1 A genetic construct of which protein coding DNA comprises  
2 introns and is designed for protein production in trans-  
3 genic animals.

4 This invention relates to the production of  
5 peptide-containing molecules.

6 Recombinant DNA technology has been used increasingly  
7 over the past decade for the production of commercially  
8 important biological materials. To this end, the DNA  
9 sequences encoding a variety of medically important  
10 human proteins have been cloned. These include  
11 insulin, plasminogen activator,  $\alpha_1$ -antitrypsin and  
12 coagulation factors VIII and IX. At present, even with  
13 the emergent recombinant DNA techniques, these proteins  
14 are usually purified from blood and tissue, an  
15 expensive and time consuming process which may carry  
16 the risk of transmitting infectious agents such as  
17 those causing AIDS and hepatitis.

18  
19 Although the expression of DNA sequences in bacteria to  
20 produce the desired medically important protein looks  
21 an attractive proposition, in practice the bacteria  
22 often prove unsatisfactory as hosts because in the  
23 bacterial cell foreign proteins are unstable and are  
24 not processed correctly.

25  
26 Recognising this problem, the expression of cloned  
27 genes in mammalian tissue culture has been attempted  
28 and has in some instances proved a viable strategy.  
29 However batch fermentation of animal cells is an  
30 expensive and technically demanding process.

31  
32 There is therefore a need for a high yield, low cost  
33 process for the production of biological substances

1 such as correctly modified eukaryotic polypeptides.  
2 The absence of agents that are infectious to humans  
3 would be an advantage in such a process.  
4

5 The use of transgenic animals as hosts has been  
6 identified as a potential solution to the above  
7 problem. WO-A-8800239 discloses transgenic animals  
8 which secrete a valuable pharmaceutical protein, in  
9 this case Factor IX, into the milk of transgenic sheep.  
10 EP-A-0264166 also discloses the general idea of  
11 transgenic animals secreting pharmaceutical proteins  
12 into their milk, but gives no demonstration that the  
13 technique is workable.  
14

15 Although the pioneering work disclosed in WO-A-8800239  
16 is impressive in its own right, it would be desirable  
17 for commercial purposes to improve upon the yields of  
18 proteins produced in the milk of the transgenic animal.  
19 For Factor IX, for example, expression levels in milk  
20 of at least 50 mcg/ml may be commercially highly  
21 desirable, and it is possible that for  $\alpha_1$ -  
22 antitrypsin higher levels of expression, such as 500  
23 mcg/ml or more may be appropriate for getting a  
24 suitably high commercial return.  
25

26 It would also be desirable if it was possible to  
27 improve the reliability of transgenic expression, as  
28 well as the quantitative yield of expression. In other  
29 words, a reasonable proportion of the initial  
30 Generation 0 (G0) transgenic animals, or lines  
31 established from them, should express at reasonable  
32 levels. The generality of the technique, in  
33 particular, is going to be limited if (say) only one in



1 a hundred animals or lines express. This is  
2 particularly the case for large animals, for which,  
3 with the techniques currently available, much time and  
4 money can be expended to produce only a small number of  
5 GO animals.

6  
7 Early work with transgenic animals, as represented by  
8 WO-A-8800239 has used genetic constructs based on cDNA  
9 coding for the protein of interest. The cDNA will be  
10 smaller than the natural gene, assuming that the  
11 natural gene has introns, and for that reason is more  
12 easy to manipulate.

13  
14 Brinster et al (PNAS 85 836-840 (1988)) have  
15 demonstrated that introns increase the transcriptional  
16 efficiency of transgenes in transgenic mice. Brinster  
17 et al show that all the exons and introns of a natural  
18 gene are important both for efficient and for reliable  
19 expression (that is to say, both the levels of the  
20 expression and the proportion of expressing animals)  
21 and is due to the presence of the natural introns in  
22 that gene. It is known that in some cases this is not  
23 attributable to the presence of tissue-specific  
24 regulatory sequences in introns, because the phenomenon  
25 is observed when the expression of a gene is redirected  
26 by a heterologous promoter to a tissue in which it is  
27 not normally expressed. Brinster et al say that the  
28 effect is peculiar to transgenic animals and is not  
29 seen in cell lines.

30  
31 It might therefore be expected that the way to solve  
32 the problems of yield and reliability of expression  
33 would be simply to follow the teaching of Brinster et

1 To illustrate this problem,  $\alpha_1$ -antitrypsin, Factor  
2 IX and Factor VIII will briefly be considered.  $\alpha_1$ -  
3 antitrypsin (AAT) comprises 394 amino acids as a mature  
4 peptide. It is initially expressed as a 418 amino acid  
5 pre-protein. The mRNA coding for the pre-protein is  
6 1.4 kb long, and this corresponds approximately to the  
7 length of the cDNA coding for AAT used in the present  
8 application (approximately 1.3 kb). The structural  
9 gene (liver version, Perlino et al, The EMBO Journal  
10 Volume 6 p.2767-2771 (1987)) coding for AAT contains 4  
11 introns and is 10.2 kb long.

12  
13 Factor IX (FIX) is initially expressed as a 415 amino  
14 acid preprotein. The mRNA is 2.8 kb long, and the cDNA  
15 that was used in WO-A-8800239 to build FIX constructs  
16 was 1.57 kb long. The structural gene is approximately  
17 34 kb long and comprises 7 introns.

18  
19 Factor VIII (FVIII) is expressed as a 2,351 amino acid  
20 preprotein, which is trimmed to a mature protein of  
21 2,332 amino acids. The mRNA is 9.0 kb in length,  
22 whereas the structural gene is 185 kb long.

23  
24 It would therefore be desirable to improve upon the  
25 yields and reliability of transgenic techniques  
26 obtained when using constructs based on cDNA, but  
27 without running into the size difficulties associated  
28 with the natural gene together with all its introns.

29  
30 It has now been discovered that high yields can be  
31 obtained using constructs comprising some but not all,  
32 of the naturally occurring introns in a gene.

33

1 According to a first aspect of the present invention,  
2 there is provided a genetic construct comprising a 5'  
3 flanking sequence from a mammalian milk protein gene  
4 and DNA coding for a heterologous protein other than  
5 the milk protein, wherein the protein-coding DNA  
6 comprises at least one, but not all, of the introns  
7 naturally occurring in a gene coding for the  
8 heterologous protein and wherein the 5'-flanking  
9 sequence is sufficient to drive expression of the  
10 heterologous protein.

11

12 The milk protein gene may be the gene for whey acid  
13 protein, alpha-lactalbumin or a casein, but the  
14 beta-lactoglobulin gene is particularly preferred.

15

16 In this specification the term "intron" includes the  
17 whole of any natural intron or part thereof.

18

19 The construct will generally be suitable for use in  
20 expressing the heterologous protein in a transgenic  
21 animal. Expression may take place in a secretory gland  
22 such as the salivary gland or the mammary gland. The  
23 mammary gland is preferred.

24

25 The species of animals selected for expression is not  
26 particularly critical, and will be selected by those  
27 skilled in the art to be suitable for their needs.  
28 Clearly, if secretion in the mammary gland is the  
29 primary goal, as is the case with preferred embodiments  
30 of the invention, it is essential to use mammals.  
31 Suitable laboratory mammals for experimental ease of  
32 manipulation include mice and rats. Larger yields may  
33 be had from domestic farm animals such as cows, pigs,

1 goats and sheep. Intermediate between laboratory  
2 animals and farm animals are such animals as rabbits,  
3 which could be suitable producer animals for certain  
4 proteins.

5

6 The 5' flanking sequence will generally include the  
7 milk protein, e.g. beta-lactoglobulin (BLG),  
8 transcription start site. For BLG it is preferred that  
9 about 800 base pairs (for example 799 base pairs)  
10 upstream of the BLG transcription start site be  
11 included. In particularly preferred embodiments, at  
12 least 4.2 kilobase pairs upstream be included.

13

14 The DNA coding for the protein other than BLG ("the  
15 heterologous protein") may code for any desired protein  
16 of interest. One particularly preferred category of  
17 proteins of interest are plasma proteins. Important  
18 plasma proteins include serine protease inhibitors,  
19 which is to say members of the SERPIN family. An  
20 example of such a protein is  $\alpha_1$ -antitrypsin. Other  
21 serine protease inhibitors may also be coded for.  
22 Other plasma proteins apart from serine protease  
23 inhibitors include the blood factors, particularly  
24 Factor VIII and Factor IX.

25

26 Proteins of interest also include proteins having a  
27 degree of homology (for example at least 90%) with the  
28 plasma proteins described above. Examples include  
29 oxidation-resistant mutants and other analogues of  
30 serine protease inhibitors such as AAT. These  
31 analogues include novel protease inhibitors produced by  
32 modification of the active site of  $\alpha_1$ -antitrypsin.  
33 For example, if the Met-358 of AAT is modified to Val,

1 this replacement of an oxidation-sensitive residue at  
2 the active centre with an inert valine renders the  
3 molecule resistant to oxidative inactivation.  
4 Alternatively, if the Met-358 residue is modified to  
5 Arg, the molecule no longer inhibits elastase, but is  
6 an efficient heparin-independent thrombin inhibitor  
7 (that is to say, it now functions like anti-thrombin  
8 III).

9  
10 The protein-coding DNA has a partial complement of  
11 natural introns or parts thereof. It is preferred in  
12 some embodiments that all but one be present. For  
13 example, the first intron may be missing but it is also  
14 possible that other introns may be missing. In other  
15 embodiments of the invention, more than one is missing,  
16 but there must be at least one intron present in the  
17 protein-coding DNA. In certain embodiments it is  
18 preferred that only one intron be present.

19  
20 Suitable 3'-sequences may be present. It may not be  
21 essential for such sequences to be present, however,  
22 particularly if the protein-coding DNA of interest  
23 comprises its own polyadenylation signal sequence.  
24 However, it may be necessary or convenient in some  
25 embodiments of the invention to provide 3'-sequences  
26 and 3'-sequences of BLG will be those of choice.  
27 3'-sequences are not however limited to those derived  
28 from the BLG gene.

29  
30 Appropriate signal and/or secretory sequence(s) may be  
31 present if necessary or desirable.

32  
33

1 According to a second aspect of the invention, there is  
2 provided a method for producing a substance comprising  
3 a polypeptide, the method comprising introducing a DNA  
4 construct as described above into the genome of an  
5 animal in such a way that the protein-coding DNA is  
6 expressed in a secretory gland of the animal.

7

8 The animal may be a mammal, expression may take place  
9 in the mammary gland, for preference. The construct  
10 may be inserted into a female mammal, or into a male  
11 mammal from which female mammals carrying the construct  
12 as a transgene can be bred.

13

14 Preferred aspects of the method are as described in  
15 WO-A-8800239.

16

17 According to a third aspect of the invention, there is  
18 provided a vector comprising a genetic construct as  
19 described above. The vector may be a plasmid, phage,  
20 cosmid or other vector type, for example derived from  
21 yeast.

22

23 According to a fourth aspect of the invention, there is  
24 provided a cell containing a vector as described above.  
25 The cell may be prokaryotic or eukaryotic. If  
26 prokaryotic, the cell may be bacterial, for example E.  
27 coli. If eukaryotic, the cell may be a yeast cell or  
28 an insect cell.

29

30 According to a fifth aspect of the invention, there is  
31 provided a mammalian or other animal cell comprising a  
32 construct as described above.

33

1 According to a sixth aspect of the invention, there is  
2 provided a transgenic mammal or other animal comprising  
3 a genetic construct as described above integrated into  
4 its genome. It is particularly preferred that the  
5 transgenic animal transmits the construct to its  
6 progeny, thereby enabling the production of at least  
7 one subsequent generation of producer animals.

8  
9 The invention will now be illustrated by a number of  
10 examples. The examples refer to the accompanying  
11 drawings, in which:

12  
13 FIGURES 1 to 10 show schematically one strategy used  
14 for elaborating fusion genes comprising DNA sequence  
15 elements from ovine beta-lactoglobulin and the gene(s)  
16 of interest, in this case  $\alpha_1$ -antitrypsin, to be  
17 expressed in the mammary gland of a mammal;

18  
19 FIGURE 11 shows a Northern blot giving the results of  
20 Example 2;

21  
22 FIGURE 12 shows an RNase protection gel, referred to in  
23 Example 2;

24  
25 FIGURE 13 shows an Immuno blot of diluted milk samples  
26 from transgenic and normal mice, referred to in Example  
27 2;

28  
29 FIGURE 14 shows a Western blot of milk whey samples  
30 from normal and two transgenic sheep (Example 3);

31  
32 FIGURE 15 shows Western blots of TCA-precipitated whey  
33 samples from normal and transgenic mice (Example 3);

1 FIGURES 16a, 16b and 17 to 20 show schematically the  
2 strategy used for elaborating a further strategy used  
3 for elaborating fusion genes comprising DNA sequence  
4 elements from ovine beta-lactoglobulin and the gene(s)  
5 of interest, in this case Factor IX, to be expressed in  
6 the mammary gland of a mammal.

7

8 EXAMPLE 1

9

10 General

11

12 Where not specifically detailed, recombinant DNA and  
13 molecular biological procedures were after Maniatis et  
14 al ("Molecular Cloning" Cold Spring Harbor (1982))  
15 "Recombinant DNA" Methods in Enzymology Volume 68,  
16 (edited by R. Wu), Academic Press (1979); "Recombinant  
17 DNA part B" Methods in Enzymology Volume 100, (Wu,  
18 Grossman and Moldgave, Eds), Academic Press (1983);  
19 "Recombinant DNA part C" Methods in Enzymology Volume  
20 101, (Wu, Grossman and Moldgave, Eds), Academic Press  
21 (1983); and "Guide to Molecular Cloning Techniques",  
22 Methods in Enzymology Volume 152 (edited by S.L. Berger  
23 & A.R. Kimmel), Academic Press (1987). Unless  
24 specifically stated, all chemicals were purchased from  
25 BDH Chemicals Ltd, Poole, Dorset, England or the Sigma  
26 Chemical Company, Poole, Dorset, England. Unless  
27 specifically stated all DNA modifying enzymes and  
28 restriction endonucleases were purchased from BCL,  
29 Boehringer Mannheim House, Bell Lane, Lewes, East  
30 Sussex BN7 1LG, UK.

31

32 [Abbreviations: bp = base pairs; Kb = kilobase pairs,  
33 AAT = alpha1-antitrypsin; BLG = beta-lactoglobulin;



1 FIX<sup>-</sup> = factor IX; E. coli = Escherichia coli; dNTPs =  
2 deoxyribonucleotide triphosphates; restriction  
3 endonucleases are abbreviated thus e.g. BamHI; the  
4 addition of -O after a site for a restriction  
5 endonuclease e.g. PvuII-O indicates that the  
6 recognition site has been destroyed]

7

8 A. PREPARATION OF CONSTRUCTIONS

9

10 Elaboration of Beta-Lactoglobulin Fusion Genes

11

12 The strategy used for elaborating fusion genes  
13 comprising DNA sequence elements from the ovine  
14 beta-lactoglobulin and the gene(s) of interest to be  
15 expressed in the mammary gland is outlined in Figures 1  
16 to 10. The approach utilises sequences derived from a  
17 lambda clone, lambdaSS-1, which contains the gene for  
18 ovine beta-lactoglobulin, and whose isolation and  
19 characterisation is outlined in International Patent  
20 Application No. WO-A-8800239 (Pharmaceutical Proteins  
21 Ltd) and by Ali & Clark (1988) Journal of Molecular  
22 Biology 199, 415-426.

23

24 The elaboration of seven constructs are described -  
25 AATB, AATA, BLG-BLG, AATC, AATD, FIXD, and DELTA-A2 in  
26 sections A1-A7 respectively. Construct AATB  
27 constitutes the primary example and the other  
28 constructs are included as comparative examples.

29

30 The nomenclature eg AATB is generally used to describe  
31 the DNA construct without its associated bacterial  
32 (plasmid) vector sequences. This form, lacking the  
33 vector sequences, corresponds to that microinjected

1 into\_fertilised eggs and subsequently incorporated into  
2 the chromosome(s) of the embryo.

3

4 **A1 AATB - Construction of pIII-15BLGgAAT**

5

6 The construct AATB is a hybrid gene which contains  
7 sequence elements from the 5'-flanking region of the  
8 ovine beta-lactoglobulin gene fused to sequences from  
9 the human gene for alpha<sub>1</sub>-antitrypsin. The features of  
10 the AATB construct are summarised in Figure 6. The  
11 sequences from the ovine beta-lactoglobulin gene are  
12 contained in a SalI - SphI fragment of about 4.2kb  
13 which contains (by inspection) a putative 'CCAAT box'  
14 (AGCCAAGTG) [see Ali & Clark (1988) Journal of  
15 Molecular Biology 199, 415-426]. In addition there are  
16 ovine BLG sequences from this SphI to a PvuII site in  
17 the 5'-untranslated region of the BLG transcription  
18 unit. The sequence of this SphI - PvuII fragment is  
19 shown in Figure 5. This latter fragment contains a  
20 putative 'TATA box' (by inspection) [see Ali & Clark  
21 (1988) Journal of Molecular Biology 199, 415-426]. The  
22 mRNA cap site / transcription start point CACTCC as  
23 determined by S1-mapping and RNase protection assays is  
24 also contained within this fragment. Beyond the fusion  
25 (PvuII-O) site are found sequences from a cDNA for  
26 human alpha<sub>1</sub>-antitrypsin and from the human  
27 alpha<sub>1</sub>-antitrypsin gene. The sequences from the 5'  
28 fusion (TaqI-O) site to the BamHI site 80 bp  
29 downstream, include the initiation ATG methionine codon  
30 for alpha<sub>1</sub>-antitrypsin. The first nucleotide  
31 (cytosine) in the AAT sequences (CGACAATG..., see  
32 Figure 5) corresponds to the last nucleotide in exon I  
33 of the AAT gene. The second nucleotide (guanosine) in

1 the AAT sequences (CGACAATG..., see Figure 5)  
2 corresponds to the first nucleotide in exon II of the  
3 AAT gene. The exclusion of intron I has been effected  
4 by using DNA from a cDNA clone p8α1ppg (see below) as  
5 the source of the first 80 bp of the AAT sequences in  
6 AATB (TaqI-0 to BamHI). The BamHI site corresponds to  
7 that found in exon II of the AAT gene. Beyond this  
8 BamHI site are approximately 6.5 kb of the human AAT  
9 gene including - the rest of exon II, intron II, exon  
10 III, intron III, exon IV, intron IV, exon V and about  
11 1.5 kb of 3'-flanking sequences. Exon V contains the  
12 AAT translation termination codon (TAA) and the  
13 putative polyadenylation signal (ATTAAA). The signal  
14 peptide for the peptide encoded by construct AATB is  
15 encoded by the AAT cDNA sequence from ATGCCGTCT to  
16 TCCCTGGCT (2 bp upstream from the BamHI site in exon  
17 II.

18

19 Plasmid pSS1tgSEα1AT

20 The subclone pSS1tgSEα1AT was constructed as described  
21 here and briefly in Example 2 of International Patent  
22 Application No. WO-A-8800239 (Pharmaceutical Proteins  
23 Ltd). This clone contains the cDNA sequences for human  
24 α<sub>1</sub>-antitrypsin inserted into the 5'-untranslated  
25 region of the ovine beta-lactoglobulin gene. The  
26 plasmid p8α1ppg containing a full length cDNA encoding  
27 an M variant of α<sub>1</sub>-antitrypsin was procured from  
28 Professor Riccardo Cortese, European Molecular Biology  
29 Laboratory, Meyerhofstrasse 1, D-6900 Heidelberg,  
30 Federal Republic of Germany (Ciliberto, Dente & Cortese  
31 (1985) Cell 41, 531-540). The strategy used in the  
32 construct BLG-AAT or pSS1tgXSTARG, now known as AATA,  
33 described in International Patent Application No.

1 WO-A-8800239 (Pharmaceutical Proteins Ltd) required  
2 that the polyadenylation signal sequence at the 3' end  
3 of the  $\alpha_1$ -antitrypsin cDNA be removed.

4

5 The polyadenylation signal was removed in the following  
6 manner. Plasmid p8 $\alpha$ lppg DNA was digested with PstI and  
7 the digestion products were separated by  
8 electrophoresis in a preparative 1% agarose gel  
9 containing 0.5  $\mu$ g/ml ethidium bromide (Sigma). The  
10 relevant fragment of about 1400 bp was located by  
11 illumination with a UV lamp (Ultra-Violet Products,  
12 Inc, San Gabriel, California, USA). A piece of  
13 dialysis membrane was inserted in front of the band and  
14 the DNA fragment subsequently electrophoresed onto the  
15 membrane. The DNA was eluted from the dialysis  
16 membrane and isolated by use of an 'ElutipD' [Sclercher  
17 and Schull, Postfach 4, D-3354, Dassel, W. Germany],  
18 employing the procedure recommended by the  
19 manufacturer. The gel purified 1400 bp PstI fragment  
20 was digested with the TaqI, electrophoresed on a  
21 preparative 1% agarose gel as described above. The  
22 TaqI - PstI fragment of approximately 300 bp comprising  
23 the 3' end of the  $\alpha_1$ -antitrypsin cDNA including the  
24 polyadenylation signal sequence was eluted and purified  
25 using an Elutip as described above, as was the TaqI -  
26 TaqI fragment of 1093 bp containing the 5' portion of  
27 the cDNA. The plasmid vector pUC8 (Pharmacia-LKB  
28 Biotechnology, Pharmacia House, Midsummer Boulevard,  
29 Central Milton Keynes, Bucks, MK9 3HP, UK) was digested  
30 with AccI and PstI, phenol/chloroform extracted and DNA  
31 recovered by ethanol precipitation. The 300 bp TaqI -  
32 PstI fragment from p8 $\alpha$ lppg was ligated using T4 DNA  
33 ligase to pUC8 cut with AccI and PstI and the ligation

1 products were used to transform E. coli strain DH-1  
2 (Gibco-BRL, PO Box 35, Trident House, Renfrew Road,  
3 Paisley PA3 4EF, Scotland, UK) to ampicillin  
4 resistance. Plasmid DNA was isolated from ampicillin  
5 resistant colonies. The correct recombinants were  
6 identified by the release of a fragment of  
7 approximately 300 bp on double digestion with AccI and  
8 PstI. The plasmid generated was called pUC8.3'AT.3.

9  
10 Plasmid pUC8.3'AT.3 was subjected to partial digestion  
11 with BstNI and the fragment(s) corresponding to  
12 linearised pUC8.3'AT.3 isolated from an agarose gel.  
13 There are seven BstNI sites in pUC.3'AT.3, five in the  
14 vector and two in the region corresponding to the  
15 3'-untranslated sequences of alpha<sub>1</sub>-antitrypsin. The  
16 BstNI linearised and gel purified DNA was digested with  
17 PstI which cuts in the pUC8 polylinker where it joins  
18 the 3' end of the cDNA insert. The PstI digested DNA  
19 was end repaired with T4 DNA polymerase in the presence  
20 of excess dNTPs and self-ligated with T4 DNA ligase.  
21 The BstNI - PstI fragment containing the  
22 polyadenylation signal sequence is lost by this  
23 procedure. The ligated material was used to transform  
24 E. coli strain DH-1 to ampicillin resistance. Plasmid  
25 DNA was isolated from ampicillin resistant colonies.  
26 The correct clone was identified by restriction  
27 analysis and comparison with pUC8.3'AT.3. The correct  
28 clone was characterised by retention of single sites  
29 for BamHI and HindIII, loss of a PstI site, and a  
30 reduction in the size of the small PvuII fragment. The  
31 correct clone was termed pB5.

32

33

1 Plasmid pB5 DNA was digested with AccI,  
2 phenol/chloroform extracted and DNA recovered by  
3 ethanol precipitation. AccI cleaved pB5 DNA was  
4 treated with calf intestine alkaline phosphatase (BCL).  
5 The reaction was stopped by adding EDTA to 10  
6 millimolar and heating at 65°C for 10 minutes. The DNA  
7 was recovered after two phenol/chloroform and one  
8 chloroform extractions by precipitation with ethanol.  
9 T4 DNA ligase was used to ligate the 1093 bp TagI -  
10 TagI fragment described above to pB5, AccI cleaved and  
11 phosphatased DNA and the ligation products were used to  
12 transform E. coli strain HB101 (Gibco-BRL) to  
13 ampicillin resistance. The identity of the correct  
14 clone (pUC8 $\alpha$ 1AT.73) was verified by restriction  
15 analysis - presence of a 909 bp HinfI fragment, a 1093  
16 bp TagI fragment, and a 87 bp BamHI fragment.

17

18 The  $\alpha_1$ -antitrypsin cDNA minus its polyadenylation  
19 signal was excised from pUC8 $\alpha$ 1AT.73 as a 1300 bp AccI -  
20 HindIII fragment and isolated from a preparative gel.  
21 The 1300 bp AccI - HindIII fragment was end-repaired  
22 with the Klenow fragment of E. coli DNA polymerase in  
23 the presence of excess dNTPs. The fragment was ligated  
24 into PvuII restricted, phosphatase treated pSS1tgSE DNA  
25 (see International Patent Application No. WO-A-8800239  
26 (Pharmaceutical Proteins Ltd) to form pSS1tgSE $\alpha$ 1AT  
27 after transforming E. coli DH-1 to ampicillin  
28 resistance.

29

30 Plasmid pIII-ISpB (see Figure 1)  
31 pSS1tgSE $\alpha$ 1AT DNA was linearised by digestion with SphI  
32 which cuts at a unique site in the plasmid in a region  
33 of DNA corresponding to the 5' flanking sequences of

1 the  $\beta$ -lactoglobulin transcription unit. The DNA was  
2 recovered after phenol/chloroform extractions by  
3 precipitation with ethanol. The SphI linearised  
4 plasmid was digested with BamHI which cuts at a unique  
5 site in the plasmid in a region of DNA corresponding to  
6 the mRNA sequences of  $\alpha_1$ -antitrypsin. The 155 bp  
7 SphI - BamHI fragment, comprising beta-lactoglobulin  
8 sequences fused to  $\alpha_1$ -antitrypsin sequences was  
9 located in a 1% agarose gel and isolated by use of an  
10 ElutipD as described above.

11  
12 The plasmid pIII-ISpB was constructed by using T4 DNA  
13 ligase to ligate the 155 bp SphI - BamHI fragment from  
14 subclone pSS1tgSEa1AT into the plasmid vector  
15 pPolyIII-I (Lathe, Vilotte & Clark, 1987, Gene 57,  
16 193-201) which had been digested with SphI and BamHI.  
17 [The vector pPolyIII-I is freely available from  
18 Dr. A. J. Clark, AFRC Institute of Animal Physiology  
19 and Genetics Research, West Mains Road, Edinburgh EH9  
20 3JQ, UK.] Clones were isolated after transforming  
21 competent E. coli DH5 $\alpha$  cells (Gibco-BRL) to ampicillin  
22 resistance. Plasmid DNA was prepared from the  
23 ampicillin resistant colonies and screened for the  
24 desired product. pIII-ISpB was confirmed as the  
25 desired product by the retention of cleavage sites for  
26 the enzymes BamHI and SphI and by the addition (when  
27 compared to the vector pPolyIII-I) of a cleavage site  
28 for the enzyme StuI. The StuI site is present in the  
29 155 bp SphI - BamHI fragment isolated from  
30 pSS-1tgSEa1AT.

31  
32 Plasmid pIII-15BLGSpB (pAT2-3) (see Figure 2)  
33 pIII-ISpB DNA was digested with the SphI and SalI.

1    SphI cuts at a unique site in the plasmid in a region  
2    of DNA corresponding to the 5' flanking sequences of  
3    the beta-lactoglobulin transcription unit. This site  
4    represents the junction between the beta-lactoglobulin  
5    sequences and the plasmid vector sequences. SalI cuts  
6    at a unique site in the plasmid in the vector  
7    polylinker sequences. The SphI/SalI digested pIII-ISpB  
8    DNA was electrophoresed on a preparative 1% agarose gel  
9    as described above. The SalI - SphI fragment of  
10   approximately 2.2 kb was eluted and purified using an  
11   Elutip as described above.

12

13   The plasmid DNA pSS-1tgXS (described in International  
14   Patent Application No. WO-A-8800239 (Pharmaceutical  
15   Proteins Ltd)) was digested with SphI and SalI and the  
16   DNA electrophoresed on a 0.9% agarose gel. The  
17   relevant SalI - SphI fragment, comprising approximately  
18   4.2 kb of DNA sequences from the 5' flanking sequences  
19   of the beta-lactoglobulin gene, was located by  
20   illumination with ultra violet light and recovered by  
21   use of an Elutip as described above.

22

23   The plasmid pIII-15BLGSpB was constructed by using T4  
24   DNA ligase to ligate the 4.2 kb SalI - SphI fragment  
25   described above into gel purified SalI - SphI digested  
26   pIII-ISpB DNA. Clones were isolated after transforming  
27   E. coli DH5 $\alpha$  (Gibco-BRL) to ampicillin resistance.  
28   Plasmid DNA was prepared from the ampicillin resistant  
29   colonies and screened for the desired product. The  
30   correct product was verified by the presence of two  
31   BamHI sites - one in the 4.2 kb fragment containing the  
32   5' flanking sequences of beta-lactoglobulin and one in  
33   the sequences corresponding to the  $\alpha_1$ -antitrypsin



1 mRNA. Cleavage of the correct product with BamHI  
2 yields two fragments including one of approximately  
3 1.75 kb which spans the cloning junctions (see  
4 Figure 2).

5  
6 Plasmid pIII-15BLGgAAT (AATB or G7) (see Figure 3)

7 An  $\alpha_1$ -antitrypsin DNA clone pATp7 was procured from  
8 Dr. Gavin Kelsey, MRC Human Biochemical Genetics Unit,  
9 The Galton Laboratory, University College London,  
10 Wolfson House, 4 Stephenson Way, London NW1 2HE, UK.  
11 This clone contains the entire  $\alpha_1$ -antitrypsin  
12 transcription unit plus 348 bp of 5' and approximately  
13 1500 bp of 3' flanking sequences as an insert of  
14 approximately 12.3 kb in the BamHI site of a plasmid  
15 vector pUC9 (Pharmacia-LKB Biotechnology, Pharmacia  
16 House, Midsummer Boulevard, Central Milton Keynes,  
17 Bucks, MK9 3HP, UK). The insert for clone pATp7 was  
18 prepared by partial BamHI and partial BqIII digestion  
19 of cosmid clone  $\alpha$ ATc1 (Kelsey, Povey, Bygrave &  
20 Lovell-Badge (1987) Genes and Development 1, 161-171).  
21 The clone pATp7 contains the gene which encodes the M<sub>1</sub>  
22 allele, which is the most frequent at the Pi locus.  
23 Most of the DNA sequence of this gene is reported by  
24 Long, Chandra, Woo, Davie & Kurachi (1984) Biochemistry  
25 23, 4828-4837.

26  
27 Plasmid DNA from pATp7 was digested with BamHI and  
28 electrophoresed in a 0.9% agarose gel. The relevant  
29 BamHI fragment, comprising approximately 6500bp of  
30  $\alpha_1$ -antitrypsin sequences from the BamHI site in  
31 exon II of this gene to a BamHI site in the 3' flanking  
32 region was located and purified by use of an Elutip as  
33 described above.

1 The -plasmid pIII-15BLGSpB (also known as AT2-3) was  
2 linearised by partial digestion with BamHI. There are  
3 two BamHI sites in this plasmid one in the sequences  
4 corresponding to the 5' flanking sequences of  
5 beta-lactoglobulin and the other in the sequences  
6 corresponding to the mRNA for alpha<sub>1</sub>-antitrypsin. The  
7 latter site is the desired site for insertion of the  
8 6500 bp BamHI fragment from pATp7. The products of the  
9 partial BamHI digestion of plasmid pIII-15BLGSpB were  
10 electrophoresed in a 0.9% agarose gel. The fragment(s)  
11 corresponding to linearised pIII-15BLGSpB were located  
12 and purified using an Elutip as described above. It is  
13 expected that this fragment preparation will contain  
14 the two possible BamHI linearised molecules. BamHI  
15 linearised, gel purified DNA was dissolved in TE (10 mM  
16 Tris.HCl, 1 mM EDTA pH 8) and treated with calf  
17 intestinal phosphatase (BCL) for 30 minutes at 37°C.  
18 The reaction was stopped by adding EDTA to 10  
19 millimolar and heating at 65°C for 10 minutes. The DNA  
20 was recovered after two phenol/chloroform and one  
21 chloroform extractions by precipitation with ethanol.

22  
23 The plasmid pIII-15BLGgAAT was constructed by using T4  
24 DNA ligase to ligate the 6500 bp BamHI fragment from  
25 pATp7 into BamHI linearised, gel purified and  
26 phosphatase treated pIII-15BLGSpB DNA. Clones were  
27 isolated after transforming E. coli DH-5 (Gibco-BRL) to  
28 ampicillin resistance. Plasmid DNA was purified from  
29 the ampicillin resistant colonies and screened for the  
30 desired product. The desired clones were characterised  
31 by restriction analysis and, in particular, by the  
32 presence of an SphI fragment of approximately 1.6 kb.  
33 Plasmid DNA was prepared for one such clone (G7) and

1 given the nomenclature pIII-15BLGgAAT (also known as  
2 AATB).

3  
4 The diagnostic 1.6kb SphI fragment was subcloned from  
5 pIII-15BLGgAAT into the SphI site of the M13 vector  
6 M13tg130 (Kieny, Lathe & Lecocq (1983) Gene 26, 91-99).  
7 The DNA sequence of 180 nucleotides from the SphI site  
8 corresponding to that in the 5' flanking region of the  
9 beta-lactoglobulin gene in a 3' direction through the  
10 fusion point of the beta-lactoglobulin and  
11 alpha<sub>1</sub>-antitrypsin sequences was determined by the  
12 chain terminator reaction using a Sequenase<sup>TM</sup> kit (USB,  
13 United States Biochemical Corporation, PO Box 22400,  
14 Cleveland, Ohio 44122, USA) according to the  
15 manufacturers instructions. The sequence of this  
16 region is given in Figure 5.

17  
18 Preparation of DNA for microinjection (see Figure 4)  
19 The β-lactoglobulin/α1-antitrypsin fusion gene insert  
20 was excised from pIII-15BLGgAAT as follows. 25-50 μg  
21 aliquots of pIII-15BLGgAAT plasmid DNA were digested  
22 with NotI and the digested material electrophoresed on  
23 a 0.6% agarose gel. The larger fragment of  
24 approximately 10.5 kb was visualised under ultra-violet  
25 light and purified using an Elutip as described above.  
26 Following ethanol precipitation of the DNA eluted from  
27 the Elutip, the DNA was further purified as follows.  
28 The DNA was extracted once with phenol/chloroform, once  
29 with chloroform and was then precipitated with ethanol  
30 twice. The DNA was washed with 70% ethanol, dried  
31 under vacuum and dissolved in TE (10 mM Tris.HCl, 1mM  
32 EDTA pH 8). All aqueous solutions used in these later  
33 stages had been filtered through a 0.22 μm filter.

1   Pipette tips were rinsed in filtered sterilised water  
2   prior to use. The DNA concentration of the purified  
3   insert was estimated by comparing aliquots with known  
4   amounts of bacteriophage lambda DNA on ethidium bromide  
5   stained agarose gels. The insert DNA was checked for  
6   purity by restriction mapping.

7

8   A2   AATA - Construction of pSS1tgXSe1AT

9

10   The construct AATA is analogous to the construct  
11   BLG-FIX or pSS1tgXSFIX described in International  
12   Patent Application No. WO-A-8800239 (Pharmaceutical  
13   Proteins Ltd). The elaboration of AATA is outlined in  
14   Example 2 of International Patent Application No.  
15   WO-A-8800239 (Pharmaceutical Proteins Ltd) as a second  
16   example of the generalised construct pSS1tgXSTARG. The  
17   first stages of the construction of AATA (ie the  
18   generation of the plasmid pSS1tgSEa1AT) are described  
19   above in section A1,

20

21   A3   BLG-BLG - Construction of pSS1tgXSDELTAclBLG (see  
22   Figures 7 and 8)

23

24   The construct is analogous to FIXA and AATA (generally  
25   designated as pSS1tgXSTARG and specifically as BLG-FIX  
26   and BLG-AAT in patent WO-A-8800239) ie, the cDNA for  
27   ovine B-lactoglobulin has been inserted into the PvuII  
28   site in the first exon of pSS1tgXSDELTAcl (see below).  
29   pSS1tgXSDELTAcl is a variant of pSS1tgXS lacking the  
30   Clal restriction site found in exon 3 which should  
31   cause a frameshift in the 2nd open reading frame in the  
32   expected bicistronic message of BLG-BLG and premature  
33   termination of any polypeptide being translated. It

1 was necessary to sabotage the 2nd open reading frame in  
2 this manner in order that the polypeptides encoded by  
3 the two open reading frames could be distinguished. In  
4 order to generate this construct a full length BLG cDNA  
5 had first to be made.

6

7 pUCBlacA

8 Two complimentary 44-mer oligonucleotides, synthesised  
9 by the Oswell DNA Service, Department of Chemistry,  
10 University of Edinburgh, and containing bases 117-159  
11 of the ovine  $\beta$ -lactoglobulin cDNA sequence (Gaye *et al*,  
12 (1986) *Biochimie* 68, 1097-1107) were annealed to  
13 generate SalI and StyI complimentary termini. The  
14 annealed oligonucleotides were then ligated using T4  
15 DNA ligase to equimolar amounts of a gel purified 457  
16 bp StyI - SmaI fragment from  $\beta$ -Lg 931 (Gaye *et al*, *op*  
17 *cit*) and gel purified pUC19 (Pharmacia-LKB  
18 Biotechnology, Pharmacia House, Midsummer Boulevard,  
19 Central Milton Keynes, Bucks, MK9. 3HP, UK) which had  
20 been digested with SalI - SmaI. After transformation  
21 of competent *E. coli* strain JM83 (see Messing (1979)  
22 Recombinant DNA Technical Bulletin, NIH Publication No.  
23 79-99, 2, No. 2 (1979), 43-48) the correct recombinant  
24 was determined by restriction analysis.

25

26 pUCBlacB

27 pUCBlacA digested with SphI and StuI was ligated to  
28 equimolar amounts of a gel purified 163 bp SphI - StuI  
29 fragment from pSS1tgSE (described in patent  
30 WO-A-8800239) using T4 DNA ligase. After  
31 transformation of competent *E. coli* strain JM83 the  
32 correct recombinant was determined by restriction  
33 analysis.

## 1    pSS1tgXSDELTAcl

2    After transformation of competent E. coli strain DL43  
3    (relevant phenotype dam<sup>-</sup>, dcm<sup>-</sup>; also called GM119, gift  
4    of Dr. D. Leach, Department of Molecular Biology,  
5    University of Edinburgh, West Mains Road, Edinburgh  
6    EH9, UK) with the plasmid pSS1tgXS plasmid DNA was  
7    isolated and digested to completion with ClaI. The DNA  
8    termini were end-repaired using the Klenow fragment of  
9    E. coli DNA polymerase in the presence of excess dNTP's  
10   prior to ligation with T4 DNA ligase in the presence of  
11   1mM hexamine cobalt chloride, 25mM KCl ([to encourage  
12   self-ligation (Rusche & Howard-Flanders (1985) Nucleic  
13   Acids Research 13, 1997-2008)]). The ligation products  
14   were used to transform competent DL43 and ClaI  
15   deficient recombinants were confirmed by restriction  
16   analysis.

17

## 18    pSS1tgSE\_BLG

19   Equimolar amounts of gel purified pSS1tgSE, digested to  
20   completion with PvuII and dephosphorylated with Calf  
21   intestinal phosphatase (BCL), were ligated to a gel  
22   purified 580 bp PvuII - SmaI fragment from pUC $\alpha$ lacB  
23   using T4 DNA ligase. After transformation of competent  
24   DH5 $\alpha$  (Gibco-BRL) the correct recombinant was confirmed  
25   by restriction analysis.

26

## 27    pSE\_BLG\_3'

28   Equimolar amounts of gel purified pSS1tgSE\_BLG digested  
29   to completion with EcoRI were ligated to 3 (~4.3-5.3)  
30   gel purified products of a partial EcoRI digestion of  
31   pSS1tgXSDELTAcl using T4 DNA ligase. After  
32   transformation of competent DH5 $\alpha$  (Gibco-BRL) the  
33   correct recombinant was identified by restriction  
34   analysis.

1 pSS1tgXSDELTAclBLG  
2 The gel purified ~3 kb SphI - HindIII fragment from  
3 pSE\_BLG\_3' was ligated to equimolar amounts of gel  
4 purified ~9.6 kb SphI-HindIII fragment from  
5 pSS1tgDELTASphXS (a derivative of pSS1tgXS lacking the  
6 SphI restriction site in the polylinker region of the  
7 vector pPoly1) using T4 DNA ligase. After  
8 transformation of competent DL43 the construct was  
9 confirmed by restriction analysis.

10

11 Isolation of DNA fragment for microinjection  
12 pSS1tgXSDELTAclBLG was digested to completion with  
13 BglIII and XbaI to pSS1tgXSDELTAclBLG was digested to  
14 completion with BglIII and XbaI to liberate the insert  
15 from the vector. The insert was recovered from an  
16 agarose gel by electroelution onto dialysis membrane  
17 (Smith (1980) Methods in Enzymology 65, 371-380).  
18 After release from the membrane the DNA was  
19 phenol/chloroform extracted, ethanol precipitated and  
20 resuspended in 100  $\mu$ l H<sub>2</sub>O ready for microinjection.

21

22 A4 AATC - Construction of pSS1pUCXSTGA.AAT (see  
23 Figure 9)

24

25 This construct contains the cDNA sequences encoding  
26 human alpha-1-antitrypsin (AAT) inserted into the  
27 second exon of the ovine  $\beta$ -lactoglobulin (BLG) gene.  
28 The aim was to determine whether or not inserting the  
29 AAT cDNA sequences at a site distant from the BLG  
30 promoter would improve the levels of expression. As  
31 such, this construct comprises the intact first exon  
32 and first intron of the BLG gene.

33

1 Since this construct contains two ATG codons (including  
2 the normal BLG initiating methionine) in the first BLG  
3 exon (ie before the sequences encoding AAT) an  
4 'in-frame' termination codon (TGA) was introduced at  
5 the junction point between BLG and AAT. This was  
6 thought necessary to prevent the production of a fusion  
7 protein between BLG and AAT. It will be noted that for  
8 AAT protein to be produced from the expected  
9 transcripts, reinitiation(at the natural initiating ATG  
10 of AAT) of transcription will have to take place after  
11 termination at this codon.

12

13 pSSltgSE.TGA

14 Two oligonucleotides (5'CTTGTGATATCG3' and  
15 5'AATTCGATATCAC3') were synthesised by the Oswell DNA  
16 Service, Department of Chemistry, University of  
17 Edinburgh. After annealing, the oligonucleotides  
18 comprise a TGA stop codon, an EcoRV site and have  
19 cohesive ends for a StyI and an EcoRI site,  
20 respectively. The annealed oligonucleotides were  
21 ligated to a gel purified StyI-EcoRI fragment of about  
22 3.2 kb isolated from pSSltgSE (pSSltgSE is described in  
23 International Patent Application No. WO-A-8800239  
24 (Pharmaceutical Proteins ltd)). This will insert these  
25 sequences at the StyI site which comprises nucleotides  
26 20-25 of BLG-exon II and generates the plasmid  
27 pSSltgSE.TGA, in which the TGA stop codon is 'in frame'  
28 with the sequences encoding BLG. Note the sequences 3'  
29 to the BLG StyI site are replaced by the  
30 oligonucleotides in this step. The ligation products  
31 were used to transform E.coli strain DH5 $\alpha$  (Gibco-BRL)  
32 to ampicillin resistance. The correct clone  
33 (pSSltgSE.TGA) was identified by restriction analysis -



1 retention of sites for EcoRI and SphI and acquisition  
2 of a site for EcoRV.

3

4 pSSltgSpX.TGA

5 pSSltgSE.TGA was cleaved with EcoRI and the cohesive  
6 termini were end-repaired by filling in with Klenow  
7 fragment of E. coli DNA polymerase in the presence of  
8 excess dNTPs. After end-repair the preparation was  
9 cleaved with SphI and the insert fragment of about  
10 800 bp (now SphI->EcoRI (blunt)) was isolated on a  
11 preparative gel. Plasmid pBJ7 (this patent, see below,  
12 section A4) was cleaved with SphI and PvuII and the  
13 larger (about 4.3 kb) fragment isolated. Note that  
14 this fragment contains the pPolyI vector sequences.  
15 The SphI-EcoRI (blunt) fragment excised from  
16 pSSltgSE.TGA was ligated using T4 DNA ligase to the  
17 SphI-PvuII fragment isolated from pBJ7 and the ligation  
18 products used to transform E. coli strain DH5 $\alpha$   
19 (Gibco-BRL) to ampicillin resistance. The correct  
20 recombinant plasmid pSSltgSpX.TGA, which contains exon  
21 I, intron I, part exon II, oligonucleotide, part exon 5  
22 and exons 6 and 7 of the BLG gene, was identified by  
23 restriction analysis.

24

25 pSSlpUCXS.TGA

26 The BLG 5' SaII - SphI fragment of about 4.2 kb was  
27 isolated from pSSltgXS (WO-A-8800239) and ligated to  
28 equimolar amounts of the SphI-XbaI insert from  
29 pSSltgSpX.TGA and SaII-XbaI cleaved plasmid vector  
30 pUC18 (Pharmacia-LKB Biotechnology, Pharmacia House,  
31 Midsummer Boulevard, Central Milton Keynes, Bucks, MK9  
32 3HP, UK). The ligation products were used to transform  
33 E. coli strain DH5 $\alpha$  (Gibco-BRL) to ampicillin

1 resistance. The correct clone, pSS1pUCXS.TGA, was  
2 identified by restriction analysis.

3

4 pSS1pUCXSAAT.TGA (AATC)

5 pSS1pUCXS.TGA contains a unique EcoRV site (derived  
6 from the oligonucleotide) inserted in the second exon  
7 which will cleave this plasmid 1 bp downstream of the  
8 'in-frame' TGA. cDNA sequences can thus be inserted  
9 into this plasmid downstream of the BLG sequences in  
10 the second exon. This is exemplified by the  
11 construction of pSS1pUCXSAAT.TGA (AATC) in which AccI -  
12 HindIII fragment derived from pUC8 $\alpha$ 1AT.73 (this patent,  
13 see Section A1 above) was inserted at the EcoRV site.  
14 Plasmid pUC8 $\alpha$ 1AT.73 (described in section A1 above) was  
15 digested with AccI and HindIII and the resulting  
16 fragment containing the alpha<sub>1</sub>-antitrypsin cDNA minus  
17 its polyadenylation signal was end-repaired using  
18 Klenow fragment of E. coli DNA polymerase in the  
19 presence of excess dNTPs. This blunt ended fragment  
20 was gel purified and ligated using T4 DNA ligase to gel  
21 purified pSS1pUCXS.TGA cleaved with EcoRV and  
22 dephosphorylated to prevent recircularisation. After  
23 transformation of competent E. coli strain DH5 $\alpha$   
24 (Gibco-BRL) with the ligation products, the correct  
25 clone was identified by restriction enzyme analysis.

26

27 A5 Construction of AATD (pBJ16) (see Figure 10)

28 This construct contains the cDNA for human  
29 alpha<sub>1</sub>-antitrypsin flanked by BLG sequences. The 5'  
30 flanking sequences include the SalI to PvuII-0 BLG  
31 sequences also present in AATA and AATB. The fusion  
32 point between the BLG and AAT sequences is in the  
33 5'-untranslated region of the BLG first exon as is the

1 case in AATA, FIXA and AATB. The 3' flanking sequences  
2 comprise exons 6 and 7 of BLG and the 3' flanking  
3 sequences of the BLG gene as far as the XbaI site.  
4 This construct contains no introns and was designed to  
5 examine whether the 5' and 3' BLG sequences described  
6 above are sufficient to direct efficient mammary  
7 specific expression of cDNAs encoding human plasma  
8 proteins as exemplified by that for AAT.

9

10 Plasmid pSS1tgSpX

11 The gel purified SphI - XbaI restriction fragment of  
12 about 6.6 kb from pSS1tgXS (described in patent  
13 WO-A-8800239) was ligated using T4 DNA ligase to gel  
14 purified pPolyI (Lathe, Vilotte & Clark, 1987, Gene 57,  
15 193-201) (also described in patent WO-A-8800239)  
16 digested with SphI and XbaI. [The vector pPolyI is  
17 freely available from Professor R. Lathe, LGME-CNRS and  
18 U184 INSERM, 11 rue Humann, 67085, Strasbourg, France.]  
19 After transformation of competent E. coli strain DHR $\alpha$   
20 (Gibco-BRL) the correct clone was identified by  
21 restriction enzyme analysis.

22

23 Plasmid pBJ5

24 The gel purified PvuII restriction fragment containing  
25 the origin of replication from pSS1tgSpX was  
26 self-ligated using T4 DNA ligase in the presence of 1mM  
27 hexamine cobalt chloride, 25mM KCl [to encourage  
28 self-ligation (Rusche & Howard-Flanders (1985) Nucleic  
29 Acids Research 13, 1997-2008)]. After transformation  
30 of competent E. coli strain DHR $\alpha$  (Gibco-BRL) the  
31 correct clone was identified by restriction enzyme  
32 analysis.

33

1 Plasmid pUCBlacA

2 See example 1 A3 for a description of pUCBlacA

3

4 Plasmid pBJ7

5 The gel purified HincII - SmaI restriction fragment

6 from pUCBlacA was ligated using T4 DNA ligase to gel

7 purified pBJ5 linearised by partial digestion with

8 SmaI. After transformation of competent E. coli strain

9 DH5 $\alpha$  (Gibco-BRL) the correct clone was identified by

10 restriction enzyme analysis.

11

12 Plasmid pBJ8

13 The gel purified PvuII restriction fragment containing

14 the origin of replication from pBJ7 was self-ligated

15 using T4 DNA ligase in the presence of 1mM hexamine

16 cobalt chloride, 25mM KCl (to encourage self-ligation

17 [Rusche & Howard-Flanders (1985) Nucleic Acids Research

18 13, 1997-2008]). After transformation into competent

19 E. coli strain DH5 $\alpha$  (Gibco-BRL) the correct clone was

20 identified by restriction enzyme analysis.

21

22 Plasmid pBJ12

23 Plasmid pUC8 $\alpha$ 1AT.73 (described in section A1 above) was

24 digested with AccI and HindIII and the resulting

25 fragment containing the  $\alpha_1$ -antitrypsin cDNA minus

26 its polyadenylation signal was end-repaired using

27 Klenow fragment of E. coli DNA polymerase in the

28 presence of excess dNTPs. This blunt ended fragment

29 was gel purified and ligated using T4 DNA ligase to gel

30 purified pBJ8 linearised with PvuII. After

31 transformation of competent E. coli strain DH5 $\alpha$

32 (Gibco-BRL) the correct clone was identified by

33 restriction enzyme analysis.

## 1 Plasmid pBJ1

2 Plasmid pSSltgSpS (described in this patent, see A7  
3 below) was digested with BglIII and end-repaired using  
4 the Klenow fragment of E. coli DNA polymerase in the  
5 presence of excess dNTPs. The blunt-ends were modified  
6 using HindIII synthetic linkers (New England Biolabs  
7 Inc, 32 Tozer Road, Beverly, MA 01915-5510, USA) and  
8 the resulting fragment self-ligated using T4 DNA ligase  
9 in the presence of 1mM hexamine cobalt chloride, 25mM  
10 KCl (to encourage self-ligation [Rusche &  
11 Howard-Flanders (1985) Nucleic Acids Research 13,  
12 1997-2008]). After transformation of competent E. coli  
13 strain DH5 $\alpha$  (Gibco-BRL) the correct clone was  
14 identified by restriction enzyme analysis.

15

## 16 Plasmid pBJ16 (AATD)

17 The gel purified HindIII - SphI fragment from pBJ1 and  
18 the gel purified SphI - XbaI fragment from pBJ12 were  
19 ligated using T4 DNA ligase to gel purified pUC19  
20 (Pharmacia-LKB Biotechnology, Pharmacia House,  
21 Midsummer Boulevard, Central Milton Keynes, Bucks, MK9  
22 3HP, UK) digested with HindIII and XbaI. After  
23 transformation of competent E. coli strain DH5 $\alpha$   
24 (Gibco-BRL) the correct clone was identified by  
25 restriction enzyme analysis.

26

27 Isolation of AAT-D fragment from pBJ16 for  
28 microinjection

29 Plasmid pBJ16 was digested with HindIII and XbaI and  
30 the resulting 8.0 kb AATD fragment was isolated from a  
31 gel using DE81 paper (Dretzen et al (1981) Analytical  
32 Biochemistry 112, 285-298). After separation from the  
33 DE81 paper the DNA was phenol/chloroform extracted,

1 ethanol precipitated and finally resuspended in TE  
2 buffer (10 mM Tris-HCl, 1mM EDTA pH 8) ready for  
3 microinjection.  
4

5 A6 FIXD - Construction of pBJ17  
6

7 The procedure of Example 1 A5 (construction of AATD) is  
8 repeated, except that the DNA sequence encoding the  
9 polypeptide of interest encodes Factor IX. A NheI -  
10 HindIII fragment comprising 1553 bp of the insert from  
11 p5'G3'CVI [see International Patent Application No.  
12 WO-A-8800239 (Pharmaceutical Proteins Ltd)] was  
13 inserted into the PvuII site of pBJ8 as described above  
14 for pBJ12.  
15

16 A7 DELTA-A2 - Construction of pSSltgXDELTA-AvaII  
17 (DELTA A2)  
18

19 This construct contains the minimum ovine  
20 beta-lactoglobulin sequences that have so far been  
21 shown in transgenic mice to result in tissue-specific  
22 expression of the protein during lactation. The  
23 complete sequence of this construct can be found in  
24 Harris, Ali, Anderson, Archibald & Clark (1988),  
25 Nucleic Acids Research 16 (in press).  
26

27 Plasmid pSSltgSpS

28 The gel purified SalI - SphI restriction fragment of  
29 approximately 4.2 kb isolated from pSSltgXS (described  
30 in patent WO-A-8800239) was ligated, using T4 DNA  
31 ligase, with equimolar amounts of gel purified pPolyI.  
32 (Lathe, Vilotte & Clark, 1987, Gene 57, 193-201)  
33 digested with SalI and SphI. [The vector pPolyI is

1 freely available from Professor R. Lathe, LGME-CNRS and  
2 U184 INSERM, 11 rue Humann, 67085 Strasbourg, France.]  
3 After transformation of competent E. coli strain DH1  
4 (Gibco-BRL) the correct clone was identified by  
5 restriction analysis.

6

7 Plasmid pSS1tgSpDELTA-AvaII

8 Plasmid pSS1tgSpS was partially digested with AvaI  
9 followed by digestion to completion with SalI. The  
10 ends of the resultant DNA fragments were end-repaired  
11 using the Klenow fragment of E. coli DNA polymerase in  
12 the presence of excess dNTPs. After ligation using T4  
13 DNA ligase in the presence of 1mM hexamine cobalt  
14 chloride, 25mM KCl [to encourage self-ligation (Rusche  
15 & Howard-Flanders (1985) Nucleic Acids Research 13,  
16 1997-2008)], the DNA was used to transform competent  
17 DH1 (Gibco-BRL). The correct AvaI deletion recombinant  
18 was confirmed by restriction analysis.

19

20 Plasmid pSS1tgXDELTA-AvaII

21 The gel purified ~800 bp SphI - BglIII fragment from  
22 pSS1tgSpDELTA-AvaII; ~6.5 kb SphI - XbaI fragment from  
23 pSS1tgXS; and pPolyI digested with BglIII - XbaI were  
24 ligated in approximately equimolar ratios using T4 DNA  
25 ligase then used to transform competent DH1  
26 (Gibco-BRL). The identity of the correct recombinant  
27 was confirmed by restriction analysis.

28

29 Isolation of DNA fragment for injection

30 pSS1tgXDELTA-AvaII was digested to completion with  
31 BglIII and XbaI to release the ~7.4 kb insert from the  
32 vector. The insert was recovered from an agarose gel  
33 using DE81 paper (Dretzen et al (1981) Analytical

1 Biochemistry 112, 295-298). After separation from the  
2 DE81 paper the DNA was phenol/chloroform extracted,  
3 ethanol precipitated and resuspended in 100  $\mu$ l TE ready  
4 for microinjection. Alternatively, the insert was  
5 recovered from an agarose gel by electroelution onto  
6 dialysis membrane (Smith (1980) Methods in Enzymology  
7 65, 371-380). After release from the membrane the DNA  
8 was phenol/chloroform extracted, ethanol precipitated  
9 and resuspended in 100  $\mu$ l H<sub>2</sub>O ready for microinjection.

10

## 11 B. CONSTRUCTION OF TRANSGENIC ANIMALS

12

### 13 MICE

14

15 Procedures are similar to those described by Hogan,  
16 Costantini and Lacy in "Manipulating the Mouse Embryo:  
17 A Laboratory Manual" Cold Spring Harbor Laboratory  
18 (1986).

19

### 20 Collection of fertilised eggs

21

22 Mice used for the collection of fertilised eggs are F<sub>1</sub>  
23 hybrids between the C57BL/6 and CBA inbred strains of  
24 mice. C57BL/6 females and CBA males are obtained from  
25 Harlan Olac Ltd (Shaw's Farm, Bicester OX6 OTP,  
26 England) and used for the breeding of F<sub>1</sub> hybrids. The  
27 mice are housed in controlled light conditions (lights  
28 on at 03.00h, lights off at 17.00h). To induce  
29 superovulation, adult female mice are injected with 5  
30 international units of Pregnant Mares Serum  
31 Gonadotropin (Cat. No. 4877, Sigma Chemical Company,  
32 Poole, Dorset, England) in 0.1 ml of distilled water,  
33 at 15.00h followed 46 to 48 hours later by injection of



1 5 international units of Human Chorionic Gonadotropin  
2 (HCG) (Cat. No. CG-10, Sigma Chemical Company, Poole,  
3 Dorset, England) in 0.1 ml of distilled water.  
4 Following HCG injection, the females are housed  
5 individually with mature C57BL/6 X CBA F<sub>1</sub> male mice for  
6 mating. The following morning, mated female mice are  
7 identified by the presence of a vaginal plug.

8  
9 Mated females are killed by cervical dislocation. All  
10 subsequent procedures are performed taking precautions  
11 to avoid bacterial and fungal contamination. Oviducts  
12 are excised and placed in M2 culture medium (Hogan,  
13 Costantini and Lacy "Manipulating the Mouse Embryo: A  
14 Laboratory Manual" Cold Spring Harbor Laboratory (1986)  
15 pp254-256). The fertilised eggs are dissected out of  
16 the ampullae of the oviducts into M2 containing  
17 300 µg/ml hyaluronidase (Type IV-S, Cat. No. H3884,  
18 Sigma Chemical Company, Poole, Dorset, England) to  
19 release the cumulus cells surrounding the fertilised  
20 eggs. Once the eggs are free of cumulus, they are  
21 washed free of hyaluronidase and, until required for  
22 injection, are kept at 37°C either in M2 in a  
23 humidified incubator, or in a drop (100 - 200 µl) of  
24 Medium No. 16 (Hogan, Costantini and Lacy "Manipulating  
25 the Mouse Embryo: A Laboratory Manual" Cold Spring  
26 Harbor Laboratory (1986) pp254-255, and 257), under  
27 mineral oil (Cat. No. 400-5, Sigma Chemical Company,  
28 Poole, Dorset, England) in an atmosphere of 95% air, 5%  
29 CO<sub>2</sub>.

30

31 Injection of DNA

32

33 The DNA to be injected is diluted to approximately

1 1.5  $\mu\text{g/ml}$  in AnalaR water (Cat. No. 10292 3C, BDH  
2 Chemicals, Burnfield Avenue, Glasgow G46 7TP,  
3 Scotland), previously sterilised by filtration through  
4 a 0.2  $\mu\text{m}$  pore size filter (Cat. No. SM 16534,  
5 Sartorius, 18 Avenue Road, Belmont, Surrey SM2 6JD,  
6 England). All micropipette tips and microcentrifuge  
7 tubes used to handle the DNA and diluent are rinsed in  
8 0.2  $\mu\text{m}$ -filtered water, to remove particulate matter  
9 which could potentially block the injection pipette.  
10 The diluted DNA is centrifuged at 12000 x g for at  
11 least 15 minutes to allow any particulate matter to  
12 sediment or float; a 20  $\mu\text{l}$  aliquot is removed from just  
13 below the surface and used to fill the injection  
14 pipettes.

15  
16 Injection pipettes are prepared on the same day they  
17 are to be used, from 15cm long, 1.0mm outside diameter,  
18 thin wall, borosilicate glass capillaries, with  
19 filament (Cat. No. GC100TF-15; Clark Electromedical  
20 Instruments, PO Box 8, Pangbourne, Reading, RG8 7HU,  
21 England), by using a microelectrode puller (Campden  
22 Instruments, 186 Campden Hill Road, London, England).  
23 DNA (approximately 1  $\mu\text{l}$ ) is introduced into the  
24 injection pipettes at the broad end; it is carried to  
25 the tip by capillary action along the filament. To  
26 prevent evaporation of water from the DNA solution,  
27 approximately 20  $\mu\text{l}$  Fluorinert FC77 (Cat. No. F4758,  
28 Sigma Chemical Company, Poole, Dorset, England) is laid  
29 over the DNA solution. The filled injection pipettes  
30 are stored at 4°C until required.

31  
32 The holding pipette (used to immobilise the eggs for  
33 microinjection) is prepared from 10cm long, 1.0mm

1 outside diameter, borosilicate glass capillaries (Cat.  
2 No. GC100-10; Clark Electromedical Instruments, PO Box  
3 8, Pangbourne, Reading RG8 7HU, England). The glass is  
4 heated over a small flame and pulled by hand to give a  
5 2 - 4 cm long section with a diameter of 80 - 120  $\mu$ m.  
6 Bends are introduced into the pipette, the glass is  
7 broken and the tip is polished using a microforge  
8 (Research Instruments, Kernick Road, Penryn TR10 9DQ,  
9 England).

10

11 A cover slip chamber is constructed in which to  
12 micromanipulate the eggs. The base of the cover-slip  
13 chamber is a 26 x 76 x (1 - 1.2)mm microscope slide  
14 (Cat. No. ML330-12, A and J Beveridge Ltd, 5 Bonnington  
15 Road Lane, Edinburgh EH6 5BP, Scotland) siliconised  
16 with 2% dimethyldichlorosilane (Cat. No. 33164 4V, BDH  
17 Chemicals, Burnfield Avenue, Glasgow G46 7TP, Scotland)  
18 according to the manufacturer's instructions; two glass  
19 supports (25 x 3 x 1 mm, cut from microscope slides)  
20 are fixed onto the slide with high vacuum silicone  
21 grease (Cat. No. 33135 3N, BDH Chemicals, Burnfield  
22 Avenue, Glasgow G46 7TP, Scotland) parallel to and  
23 approximately 2mm from the long sides of the slide,  
24 half way along the length of the slide. A further two  
25 glass supports are fixed on top of the first pair, and  
26 the top surface is smeared with silicone grease.  
27 300  $\mu$ l of medium M2 are pipetted into the space between  
28 the supports, and a 22 x 22 mm cover-slip (Cat. No.  
29 ML544-20, A and J Beveridge Ltd, 5 Bonnington Road  
30 Lane, Edinburgh EH6 5BP, Scotland) is lowered onto the  
31 supports, a seal being formed by the grease.  
32 Dow-Corning fluid (50 cs) (Cat. No. 63006 4V, BDH  
33 Chemicals, Burnfield Avenue, Glasgow G46 7TP, Scotland)

1 is pipetted into the open ends of the chamber, to cover  
2 the medium.

3  
4 Batches of eggs (30 to 100) are placed into a  
5 cover-slip chamber for manipulation. The chamber is  
6 mounted on the microscope (Diaphot, Nikon (UK) Ltd,  
7 Haybrooke, Telford, Shropshire, England) which has 4x  
8 bright field, 10x phase contrast and 40x differential  
9 interference contrast (DIC) objectives, and 10x  
10 eyepieces. Mechanical micromanipulators (Cat. Nos.  
11 520 137 and 520 138, E. Leitz (Instruments) Ltd, 48  
12 Park Street, Luton, England) are mounted adjacent to  
13 the microscope and are used to control the positions of  
14 the holding and injection pipettes.

15  
16 The holding pipette and DNA-containing injection  
17 pipette are mounted in modified instrument tubes (Cat.  
18 No. 520 145, E. Leitz (Instruments) Ltd, 48 Park  
19 Street, Luton, England) which are in turn mounted onto  
20 the micromanipulators via single unit (Cat. No.  
21 520 142, E. Leitz (Instruments) Ltd, 48 Park Street,  
22 Luton, England) and double unit (Cat. No. 520 143, E.  
23 Leitz (Instruments) Ltd, 48 Park Street, Luton,  
24 England) instrument holders, respectively. The  
25 instrument tubes are modified by gluing onto Clay Adams  
26 "Intramedic" adapters (2.0-3.5 mm tubing to female  
27 Luer, Cat. No. 7543D, Arnold R. Horwell Ltd, 2  
28 Grangeway, Kilburn High Road, London NW6 2BP, England),  
29 which are used to connect the instrument tubes to  
30 approximately 2 metres of polythene tubing (1.57 mm  
31 inside diameter, 2.9 mm outside diameter, Cat. No.  
32 F21852-0062, R.B. Radley & Co, Ltd, London Road,  
33 Sawbridgeworth, Herts CM21 9JH, England), further

1 "Intramedic" adapters are connected to the other ends  
2 of the polythene tubing to facilitate connection to the  
3 syringes used to control the holding and injection  
4 pipettes.

5

6 Injection is controlled using a 20ml or a 100ml glass  
7 syringe (Cat. Nos. M611/20 and M611/31, Fisons, Bishop  
8 Meadow Road, Loughborough LE11 0RG, England), the  
9 plunger of which is lightly greased with high vacuum  
10 silicone grease (Cat. No. 33135 3N, BDH Chemicals,  
11 Burnfield Avenue, Glasgow G46 7TP, Scotland).

12

13 Holding of eggs is controlled with an Agla micrometer  
14 syringe (Cat. No. MS01, Wellcome Diagnostics, Temple  
15 Hill, Dartford DA1 5AH, England), which is fitted with  
16 a light spring around the plunger. The Agla syringe is  
17 connected via a 3-way stopcock (Cat. No. SYA-580-L),  
18 Gallenkamp, Belton Road West, Loughborough LE11 0TR,  
19 England), to the "Intramedic" adapter, the third port  
20 of the stopcock is connected to a reservoir of  
21 Fluorinert FC77 (Cat. No. F 4758, Sigma Chemical  
22 Company, Poole, Dorset, England), which fills the Agla  
23 syringe, polythene tubing, instrument tube and holding  
24 pipette.

25

26 The tip of the injection pipette is broken off against  
27 the holding pipette, to increase the tip diameter to a  
28 size which allows free passage of the DNA solution and  
29 which is small enough to allow injection without lethal  
30 damage to the eggs ( $\leq 1 \mu\text{m}$ ). The flow of DNA through  
31 the pipette tip is checked by viewing under phase  
32 contrast conditions whilst pressure is applied to the  
33 injection syringe (the DNA solution will appear as a  
34 bright plume emerging from the tip of the pipette).

1 One by one, fertilised eggs are picked up on the  
2 holding pipette, and one or both pronuclei brought into  
3 the same focus as the injection pipette (using the 40x  
4 objective and DIC conditions; the correction ring on  
5 the objective is adjusted for optimum resolution). The  
6 injection pipette is inserted into one of the  
7 pronuclei, avoiding the nucleoli, pressure is applied  
8 to the injection syringe and once swelling of the  
9 pronucleus is observed, pressure is released and the  
10 injection pipette is immediately withdrawn. When  
11 pipettes block, the blockage may be cleared by  
12 application of high pressure on the injection syringe  
13 or by breaking off a further portion of the tip. If  
14 the blockage cannot be cleared, or if the pipette tip  
15 becomes dirty, the pipette is replaced.

16  
17 After injection, the eggs are cultured overnight in  
18 medium No. 16 under oil in an atmosphere of 5% CO<sub>2</sub>.  
19 Eggs which cleave to two cells during overnight culture  
20 are implanted into pseudopregnant foster mothers.

21  
22 Random-bred albino (MF1, Harlan Olac Ltd, Shaw's Farm,  
23 Bicester, OX6 OTP, England) female mice are mated with  
24 vasectomised (Hogan, Costantini and Lacy, "Manipulating  
25 the Mouse Embryo: A Laboratory Manual" Cold Spring  
26 Harbor Laboratory (1986); Rafferty, "Methods in  
27 experimental embryology of the mouse", The Johns  
28 Hopkins Press, Baltimore, USA (1970)) MF1 male mice.  
29 The matings are performed one day later than those of  
30 the superovulated egg donors. MF1 females which have a  
31 detectable vaginal plug the following morning are used  
32 as foster mothers. The ideal weight of foster mothers  
33 is 25 to 30g. Each foster mother is anaesthetised by

1 intraperitoneal injection of Hypnorm/Hypnovel (10  $\mu$ l/g  
2 body weight) at 2/3 the concentration recommended by  
3 Flecknell (Veterinary Record, 113, 574) (Hypnorm: Crown  
4 Chemical Co, Ltd, Lamberhurst, Kent TN3 8DJ, England;  
5 Hypnovel: Roche Products Ltd, PO Box 8, Welwyn Garden  
6 City, Herts AL7 3AY, England) and 20 to 30 2-cell eggs  
7 are transferred into one oviduct by the method  
8 described by Hogan, Costantini and Lacy ("Manipulating  
9 the Mouse Embryo: A Laboratory Manual" Cold Spring  
10 Harbor Laboratory (1986)). As an option, to minimise  
11 bleeding from the ovarian bursa, 2  $\mu$ l of 0.01% (w:v)  
12 epinephrine bitartrate (Cat. No. E4375, Sigma Chemical  
13 Company, Poole, Dorset, England) dissolved in distilled  
14 water is applied to the bursa a few minutes before  
15 tearing it. Foster mothers are allowed to deliver  
16 their offspring naturally unless they have not done so  
17 by 19 days after egg transfer, in which case the pups  
18 are delivered by hysterectomy, and are fostered.  
19 Following normal mouse husbandry, the pups are weaned  
20 at 3 to 4 weeks of age and housed with other mice of  
21 the same sex only.

22  
23 Transgenic female mice may be used for the breeding of  
24 subsequent generations of transgenic mice by standard  
25 procedures and/or for the collection of milk and RNA.  
26 Transgenic male mice are used to breed subsequent  
27 generations of transgenic mice by standard procedures.  
28 Transgenic mice of subsequent generations are  
29 identified by analysis of DNA prepared from tails, as  
30 described below.

31  
32  
33

1 SHEEP

2

3 The generation of transgenic sheep is described in  
4 detail in International Patent Application No.  
5 WO-A-8800239 (Pharmaceutical Proteins Ltd) and by  
6 Simons, Wilmut, Clark, Archibald, Bishop & Lathe (1988)  
7 Biotechnology 6, 179-183.

8

9 C. IDENTIFICATION OF TRANSGENIC INDIVIDUALS

10

11 MICE

12

13 When the pups are at least 4 weeks of age, a biopsy of  
14 tail is taken for the preparation of DNA. The pups are  
15 anaesthetised by intraperitoneal injection of  
16 Hypnorm/Hypnovel (10  $\mu$ l/g body weight) at 1/2 the  
17 concentration recommended by Flecknell (Veterinary  
18 Record, 113, 574). Once anaesthetised, a portion of  
19 tail (1 to 2 cm) is removed by cutting with a scalpel  
20 which has been heated in a Bunsen flame; the hot blade  
21 cauterises the wound and prevents bleeding.

22

23 The tail segments are digested with proteinase  
24 K 200  $\mu$ g/ml (Sigma) in tail buffer [0.3 M NaAcetate  
25 (not titrated), 10 mM Tris-HCl pH 7.9, 1 mM EDTA pH  
26 8.0, 1% SDS] overnight with shaking at 37°C. The  
27 following day the digests are vortexed briefly to  
28 disaggregate the debris. Aliquots of digested tail are  
29 phenol/chloroform extracted once, chloroform extracted  
30 once and then DNA is recovered by precipitation with an  
31 equal volume of isopropanol.

32

33



1 'Tail DNA' is digested with restriction enzyme(s), and  
2 subjected to agarose gel electrophoresis. The  
3 separated DNA is then 'Southern' blotted to Hybond<sup>TM</sup> N  
4 (Amersham) nylon membranes as described in the Amersham  
5 Handbook 'Membrane transfer and detection methods'  
6 (Pl/162/86/8 published by Amersham International plc,  
7 PO Box 16, Amersham, Buckinghamshire HP7 9LL, UK). DNA  
8 bound to the membranes is probed by hybridisation to  
9 appropriate <sup>32</sup>P labelled DNA sequences (eg the  
10 construct DNAs). The DNA probes are labelled with <sup>32</sup>P  
11 by nick-translation as described in 'Molecular Cloning:  
12 a Laboratory Manual' (1982) by Maniatis, Fritsch and  
13 Sambrook, published by Cold Spring Harbor Laboratory,  
14 Box 100, Cold Spring Harbor, USA. Alternatively DNA  
15 probes are labelled using random primers by the method  
16 described by Feinberg and Vogelstein (1984) Analytical  
17 Biochemistry 137, 266-267. Briefly: The plasmid or  
18 phage is cleaved with the appropriate restriction  
19 enzymes and the desired fragment isolated from an  
20 agarose gel. The labelling reaction is carried out at  
21 room temperature by adding the following reagents in  
22 order: H<sub>2</sub>O, 6 µl OLB\*, 1.2 µl BSA, DNA (max. 25 ng),  
23 4 µl <sup>32</sup>P labelled dCTP (PB10205, Amersham plc, Amersham  
24 UK), 1 µl (1 unit) Klenow Polymerase (BCL) to a final  
25 volume of 30 µl.

26  
27 \*OLB comprises solution A: 625 µl 2M Tris, pH 8.0 + 25  
28 µl 5M MgCl<sub>2</sub> + 350 µl H<sub>2</sub>O + 18 µl 2-mercaptoethanol  
29 (Sigma); solution B, 2M HEPES (Sigma), titrated to pH  
30 6.6 with NaOH; solution C, Hexa deoxyribonucleotides  
31 (Pharmacia-LKB Biotechnology Cat. No. 27-2166-01). The  
32 labelling reaction is allowed to run overnight and then  
33 the reaction stopped by the addition of 70 µl stop

1 solution (20 mM NaCl, 20 mM Tris pH 7.5, 2mM EDTA,  
2 0.25% SDS, 1  $\mu$ M dCTP). Incorporation is assessed by  
3 TCA precipitation and counting Cerenkov emission.

4  
5 Hybridisations are carried out in sealed plastic bags  
6 by a modification of the procedure described by Church  
7 and Gilbert (1984). Proceedings of the National  
8 Academy of Sciences (USA) 81, 1991-1995. Briefly: the  
9 probe is used at a concentration of  $1.5 \times 10^6$  Cerenkov  
10 counts/ml of hybridisation buffer (HB: 0.5M sodium  
11 phosphate pH 7.2, 7% SDS, 1mM EDTA). Firstly, the  
12 membrane is prehybridised for 5 minutes in HB (15ml of  
13 buffer per 20 cm<sup>2</sup> membrane) in the plastic bag at 65°C.  
14 The probe is denatured by boiling and added to the same  
15 volume of fresh HB. The plastic bag is cut open and  
16 the prehybridisation solution drained and then the HB +  
17 probe added and the bag re-sealed. The bag and  
18 contents are incubated overnight on a rotary shaker at  
19 65°C. After hybridisation the membrane is washed in 40  
20 mM sodium phosphate, 1% SDS and 1mM EDTA three times  
21 for ten minutes at 65°C and then a final wash is  
22 carried out for 15-30 minutes at this temperature.  
23 Washing is monitored with a hand-held Geiger counter.  
24 The stringency of the washings may be adjusted  
25 according to the particular needs of the experiment.  
26 After the last wash the membrane is blotted dry and  
27 then placed on a dry piece of Whatman filter paper and  
28 wrapped in Saran-wrap. The membrane is exposed to  
29 X-ray film (Agfa CURIX RP-1) using an X-ray cassette at  
30 - 70°C for one or more days.

31

32 By comparison with known amounts of construct DNA  
33 treated in the same manner DNA from transgenic

1 individuals can be identified and the number of copies  
2 of the construct DNA which have been integrated into  
3 the genome can be estimated.

4

5 The same methods are used to identify transgenic  
6 offspring of the founder transgenic individuals.

7

8 SHEEP

9

10 The identification of transgenic sheep is described in  
11 detail in International Patent Application No.  
12 WO-A-8800239 (Pharmaceutical Proteins Ltd).

13

14 D. ANALYSIS OF EXPRESSION - METHODS

15

16 Collection of Mouse Milk

17

18 Female mice (at least 7 weeks of age) are housed  
19 individually with adult male mice for mating. After 17  
20 days, the male mice are removed from the cage and the  
21 female mice are observed daily for the birth of  
22 offspring. Milk and/or RNA are collected 11 days after  
23 parturition.

24

25 For the collection of milk, the pups are separated from  
26 the lactating female mice to allow the build-up of milk  
27 in the mammary glands. After at least 3 hours, 0.3  
28 international units of oxytocin (Sigma, Cat. No.  
29 O 4250) in 0.1 ml of distilled water are administered  
30 by intraperitoneal injection, followed after 10 minutes  
31 by intraperitoneal injection of Hypnorm/Hypnovel  
32 anaesthetic (10  $\mu$ l/g body weight) at 2/3 the  
33 concentration recommended by Flecknell (Veterinary

1 Record, 113, 574). When fully anaesthetised, the  
2 mammary glands are massaged to expel milk, which is  
3 collected in 50  $\mu$ l capillary tubes (Drummond Microcaps,  
4 Cat. No. PP600-78, A and J Beveridge Ltd, 5 Bonnington  
5 Road Lane, Edinburgh EH6 5BP, Scotland).

6  
7 Mouse milk is diluted 1:5 in distilled water and  
8 centrifuged in an Eppendorf 5415 centrifuge (BDH) to  
9 remove fat. To make whey, 1.0 M HCl was added to give  
10 a final pH of 4.5, thus precipitating the caseins which  
11 were then removed by centrifugation in an Eppendorf  
12 5415 centrifuge. Diluted milk or whey samples were  
13 solubilised by boiling in loading buffer prior to  
14 discontinuous SDS polyacrylamide gel electrophoresis  
15 (Laemmli (1970) Nature 227, 680-684) and immunoblotting  
16 analysis (Khyse-Anderson (1984) Journal of Biochemical  
17 and Biophysical Methods 10, 203-209). Human  
18  $\alpha_1$ -antitrypsin (AAT) was identified on immunoblot  
19 filters by using goat-anti-AAT serum [Protein Reference  
20 Unit, Royal Hallamshire Hospital, Sheffield S10 2JF]  
21 and anti-sheep/goat IgG serum conjugated to horseradish  
22 peroxidase [Scottish Antibody Production Unit, Glasgow  
23 and West of Scotland Blood Transfusion Service, Law  
24 Hospital, Carlisle, Lanarkshire ML8 5ES].

25  
26 Amounts of human  $\alpha_1$ -antitrypsin (AAT) in mouse milk  
27 were measured by using LC-Partigen radial  
28 immunodiffusion plates [Behring Diagnostics, Hoescht UK  
29 Ltd, 50 Salisbury Road, Hounslow, Middlesex TW4 6JH].  
30 The radial immunodiffusion (RID) method, which is  
31 designed to detect AAT in body fluids in the  
32 concentration range 8 - 125  $\mu$ g/ml, was carried out  
33 according to the manufacturers instructions. Three

1 dilutions of standard human serum [LC-V, Behring  
2 Diagnostics] were prepared in phosphate buffered saline  
3 (PBS) to give AAT concentrations which fell within the  
4 detection range for the assay.

5  
6 Test milk samples were diluted 1:5 in distilled water  
7 and defatted by spinning briefly in an Eppendorf 5415  
8 centrifuge (BDH). The following control experiment was  
9 carried out in order to assess the effect of the milk  
10 environment on the detection of AAT (the method is  
11 primarily designed for measuring AAT in blood serum).  
12 Milk samples from non-transgenic mice were assayed with  
13 and without defined amounts of added AAT. Samples  
14 (20  $\mu$ l) were loaded into the wells and the plates left  
15 open for 10 - 20 minutes. The plates were then sealed  
16 with the plastic lids provided and left to stand at  
17 room temperature. The diameters of the precipitation  
18 zones were measured after a diffusion time of 2 - 3  
19 days, using a low power binocular microscope fitted  
20 with a lens graticule. At least three independent  
21 readings were recorded and the average measurement (mm)  
22 calculated and squared ( $\text{mm}^2$ ). A calibration curve  
23 plotting zone measurement squared against AAT  
24 concentration was constructed using the values obtained  
25 with the dilutions of standard human serum. This  
26 linear graph was used to calculate the AAT  
27 concentrations in the test samples.

28

29 Preparation of RNA

30

31 RNA may be prepared from mice immediately after milking  
32 or from mice which have not been milked. The lactating  
33 female mouse is killed by cervical dislocation and

1 tissues excised, taking care to avoid cross-  
2 contamination of samples. The procedure is based on  
3 the protocol described by Chirgwin, Przybyla, MacDonald  
4 and Rutter (1979) Biochemistry 18, 5294-5299.

5  
6 The tissue of interest is dissected and placed in 4 ml  
7 of a 4 M solution of Guanadine Thiocyanate in a sterile  
8 30 ml disposable plastic tube. The tissue is  
9 homogenised using an Ultra-Turrax<sup>R</sup> homogeniser at full  
10 speed for 30 - 45 seconds at room temperature. The  
11 homogenate is layered onto a 1.2 ml, 5.7 M CsCl  
12 solution in a 5 ml polyallomer ultracentrifuge tube  
13 (Sorvall Cat. 03127; Du Pont (UK) Ltd, Wedgwood Way,  
14 Stevenage, Hertfordshire SG1 4QN, UK). The RNA is  
15 pelleted through the cushion of CsCl by centrifuging at  
16 36,000 rpm for 12 hrs at 20°C using a Sorvall AH650 or  
17 Beckman SW50.1 swing-out rotor in a Beckman L80  
18 ultracentrifuge (Beckman Instruments (UK) Ltd, Progress  
19 Road, Sands Industrial Estate, High Wycombe, Bucks HP12  
20 4JL, UK). After centrifugation the supernatant is  
21 removed with sterile disposable plastic 5 ml pipettes  
22 and the tube is then very carefully drained. The RNA  
23 which should be visible as an opalescent pellet at the  
24 bottom of the tube is resuspended in 2 ml of 7.5 M  
25 Guanidine Hydrochloride with vigorous vortexing.  
26 Resuspension may take 15 minutes or longer. The  
27 preparation is transferred to a 15 or 30 ml  
28 heat-sterilised Corex<sup>TM</sup> (Du Pont) centrifuge tube and  
29 precipitated by the addition of 50 µl of 1M acetic acid  
30 and 1ml of 100% ethanol and incubation overnight at  
31 -20°C. The RNA is pelleted using a Sorvall SS34 rotor  
32 (Du Pont) in a Sorvall RCB5 refrigerated centrifuge  
33 (Du Pont) at 10,000 rpm for 10 minutes at 2°C. The RNA

1 pellet is resuspended in 2 ml of diethylpyrocarbonate  
2 (Sigma) (DEPC)-treated distilled water by vortexing.  
3 The RNA is re-precipitated by the addition of 600  $\mu$ l of  
4 1M sodium acetate (DEPC-treated) and 3 volumes of 100%  
5 ethanol, resuspended in DEPC treated water and again  
6 precipitated. After the second precipitation from DEPC  
7 water the RNA pellet is resuspended in DEPC water to  
8 the desired final volume (usually 100  $\mu$ l - 500  $\mu$ l).  
9 The concentration of RNA is determined spectro-  
10 photometrically ( $OD_{260nm} = 1$  corresponds to 40  $\mu$ g/ml).  
11 RNA preparations are stored frozen at  $-70^{\circ}\text{C}$ .  
12

### 13 Analysis of RNA

14

15 The expression of the introduced transgene was  
16 investigated in a number of different tissues by  
17 'Northern' blotting of the RNA samples prepared by the  
18 procedure described above. Aliquots (10  $\mu$ g-20  $\mu$ g) of  
19 total RNA were denatured and separated in denaturing  
20 MOPS/formaldehyde (1 - 1.5%) agarose gels and  
21 transferred to Hybond<sup>TM</sup> N (Amersham) nylon membranes as  
22 described in the Amersham Handbook 'Membrane transfer  
23 and detection methods' (PI/162/86/8 published by  
24 Amersham International plc, PO Box 16, Amersham,  
25 Buckinghamshire HP7 9LL, UK). The RNA bound to the  
26 membranes is probed by hybridisation to appropriate  $^{32}\text{P}$   
27 labelled DNA sequences (eg encoding BLG, FIX or AAT).  
28 The labelling and hybridisation procedures are  
29 described in section 1C above.  
30

31 In some cases RNA transcripts were detected using an  
32 RNase protection assay. This allows the determination  
33 of the transcriptional start point of the gene. The

1 procedure essentially follows that described by Melton,  
2 Krieg, Rebagliati, Maniatis, Zinn and Green (1984)  
3 Nucleic Acids Research 18, 7035-7054. For example, for  
4 FIX a 145bp SphI-EcoRV fragment from pSltgXSFIX  
5 (WO-A-8800239) which spans the 5' fusion point of BLG  
6 and FIX was cloned into SphI-SmaI cleaved pGEM4  
7 (ProMega Biotec, 2800 South Fish Hatchery Road,  
8 Madison, Wisconsin 53791-9889, USA). A 192 nucleotide  
9 long  $^{32}\text{p}$  labelled, antisense RNA transcript was  
10 generated using SP6 polymerase was used in the RNase  
11 protection assays. After annealing the samples were  
12 digested with RNAase A (BCL) (40  $\mu\text{g/ml}$ ) and RNase  
13 T1(BCL) (2  $\mu\text{g/ml}$ ) at 37°C for one hour.  
14 Phenol/Chloroform purified samples were electrophoresed  
15 on 8% polyacrylamide/urea sequencing gels.

16  
17 EXAMPLE 2: EXPRESSION OF THE AATB CONSTRUCT IN  
18 TRANSGENIC MICE

19  
20 The efficient expression of a human plasma protein in  
21 the milk of transgenic mice is exemplified by construct  
22 AATB. The details of the construction of AATB are  
23 given in Example 1. Briefly AATB contains the genomic  
24 sequences for the human (liver)  $\alpha_1$ -antitrypsin gene  
25 minus intron 1, fused to the promoter of the ovine  
26 beta-lactoglobulin gene. The fusion point is in the  
27 5'-untranslated region of the BLG gene. It was  
28 anticipated that the presence of the AAT introns would  
29 enhance the levels of expression of the construct. The  
30 large first AAT intron (ca. 5 kb) was omitted in order  
31 to facilitate the DNA manipulation of the construct and  
32 to determine whether all the AAT introns were required  
33 for efficient expression.



1 Unless otherwise stated the analyses of expression are  
2 tabulated. '+' indicates expression as determined by  
3 the presence of the appropriate mRNA transcript  
4 (detected by Northern blotting) or protein (as detected  
5 by radial immunodiffusion (RID) or immunoblotting  
6 (Western blotting)). '-' indicates that the expression  
7 was not detected.

8

9 Transgenic mice carrying the AATB construct

10

11 The AATB construct described in Example 1 was used to  
12 generate transgenic mice by the methods outlined in  
13 Example 1. AATB construct DNA was microinjected into  
14 fertilised mouse eggs on 7 occasions between August  
15 1987 and June 1988. A total of 993 eggs were injected  
16 of which 747 were transferred to recipient  
17 pseudo-pregnant mice. A total of 122 pups were weaned.  
18 Analysis of DNA prepared from tail biopsies, as  
19 described in Example 1C, revealed that of these 122  
20 generation zero (G0) pups 21 carried the AATB construct  
21 as a transgene (see Table 1). These transgenic mice  
22 had between 1 and >20 copies of the AATB construct  
23 integrated into their genome.

24

25 The following policy was adopted for the study of the  
26 expression of the AATB transgene. Where a founder  
27 transgenic G0 individual was male, he was mated to  
28 non-transgenic females to generate G1 offspring. Tail  
29 DNAs from G1 individuals were examined to determine  
30 whether they had inherited the transgene. Female  
31 transgenic G1 mice were used for the analysis of  
32 expression of the AATB transgene by the methods  
33 described in Example 1D. Where a founder transgenic G0

1 individual was female she was used directly for the  
 2 analysis of expression as described in Example 1D. The  
 3 adoption of this policy meant that lines of mice were  
 4 only established where the founder GO animal was male.  
 5 The transmission of the transgenes to subsequent  
 6 generations has also only been determined where the  
 7 founder GO mouse was male. Transmission data for four  
 8 AATB GO males is given in Table 1.

9  
 10 TABLE 1: Mice carrying the AATB construct as a  
 11 transgene.

14	Animal	Sex	Copy	Transmission data	
15	ID		Number	No. of offspring/No. transgenic	
17	AATB15	male	2-5	25	8
18	AATB17	male	10-15	26	16
19	AATB26	male	≥20	34	5
20	AATB28	male	2-5	22	12
21	AATB44	female	15		
22	AATB45	female	1-2		
23	AATB65	female	2-3		
24	AATB69	female	1-2		
25	AATB105	female	20		

26  
 27 Analysis of expression

28  
 29 Fifteen G1 females have been examined for expression of  
 30 the AATB transgene, 8 by protein analysis of milk and 7  
 31 by RNA analysis by the methods described in Example 1.  
 32 A further 5 GO females have been examined by both  
 33 protein analysis of milk and RNA analysis. A total of

1 9 different transgenic mice or mouse-lines were  
2 examined.

3

#### 4 RNA Analysis

5 RNAs isolated from the following tissues were examined  
6 for the presence of AATB transcripts - mammary gland,  
7 liver, kidney, spleen, salivary gland and heart. Total  
8 RNA samples (10  $\mu$ g) from these tissues were analysed by  
9 Northern blotting. A representative Northern blot is  
10 presented as Figure 11 [Lanes 1 & 2, and 3 & 4 contain  
11 mammary (M) and liver (L) samples from control mice;  
12 lanes 5 - 9, AATB26.1 mammary (M), liver (L), kidney  
13 (K), spleen (Sp) and salivary (Sa) RNA samples; lanes  
14 10 - 14, AATB17.3 mammary (M), liver (L), kidney (K),  
15 spleen (Sp) and salivary (Sa) RNA samples. The AAT  
16 transcript of approximately 1400 nucleotides is  
17 arrowed]. The human AAT cDNA probe, p8 $\alpha$ lppg,  
18 cross-hybridises with endogenous mouse AAT transcripts  
19 in liver RNA samples. The presence of AAT transcripts  
20 in salivary samples from AATB26.1 and AATB17.3 do not  
21 result from contamination with liver or mammary  
22 material as proved by re-probing the filters with  
23 liver-specific and salivary-specific probes. The  
24 results of this analysis are summarised in Table 2.

25

26

27

28

29

30

31

32

33

1 TABLE 2: Summary of RNA analysis for AATB transgenic  
2 mice.

3	4 Animal ID	5 Generation	6 Tissue (presence/absence of AATB transcripts)					
			Mam.	Liver	Kid.	Spl.	Saliv.	Heart
7	AATB15.2	G1	++	?	-	-	-	-
8	AATB15.13	G1	-	?	-	-	-	NT
9	AATB17.3	G1	+	?	-	-	+	NT
10	AATB17.20	G1	+	-	-	-	+	NT
11	AATB26.1	G1	-	-	-	-	+	NT
12	AATB26.28	G1	-	?	-	-	+	-
13	AATB28.3	G1	-	?	-	-	-	NT
14	AATB28.21	G1	-	?	-	-	-	NT
15	AATB44	GO	+	?	-	-	-	-
16	AATB45	GO	+	?	-	-	-	-
17	AATB65	GO	+	?	-	-	-	-
18	AATB69	GO	+	?	-	-	-	-
19	AATB105	GO	-	?	-	-	+	-

20  
21 [Mam. = mammary gland; Kid. = kidney; Spl. = spleen;  
22 Saliv. = salivary gland; nd = not detected; NT = not  
23 tested]

24 \* presence only detected in poly A+ RNA

25 ? background from endogenous mouse AAT transcripts in  
26 liver precluded an unambiguous determination of whether  
27 there were AATB transcripts present.

28  
29 In order to confirm that the transcripts observed were  
30 being initiated at the beta-lactoglobulin start site in  
31 the AATB constructs, RNAs isolated from the mammary  
32 gland of mouse AATB17.20 and from the salivary gland of  
33 mouse AATB26.1 were examined by an RNase protection

1 assay as described in Example 1D. RNAs isolated from  
2 the liver (AATB17.20 & AATB26.1) and from the mammary  
3 gland (AATB26.1) of these mice were also examined by  
4 RNase protection, as were RNAs from non-transgenic  
5 liver, mammary gland and salivary gland. The  
6 anti-sense probe was produced by transcribing a pGEM  
7 vector (Promega Biotec, 2800 South Fish Hatchery Road,  
8 Madison, Wisconsin 53791-9889) containing a 155 bp SphI  
9 - BamHI fragment derived from the 5' end of the AATA  
10 construct. This 155 bp fragment is identical to that  
11 found in AATB (see pIII-ISpB, Example 1A). Annealing  
12 was carried out under standard conditions and the  
13 hydrolysis of single-stranded RNA performed with RNaseA  
14 and RNaseT1(BCL). A sense transcript was also  
15 transcribed and various amounts of this transcript  
16 included along with 20  $\mu$ g samples of control RNA to  
17 provide an estimation of steady state mRNA levels. A  
18 representative RNase protection gel is shown in Figure  
19 12 [Lanes 1 & 2, AATB17.20 20  $\mu$ g and 10  $\mu$ g total  
20 mammary RNA; lanes 3, 4, 5 & 6, 1000 pg, 200 pg, 100 pg  
21 & 50 pg of control sense transcript; lanes 7 & 8,  
22 AATB26.1 20  $\mu$ g & 10  $\mu$ g total salivary RNA; lanes 9, 10  
23 & 11, 5  $\mu$ g aliquots of mammary polyA+ RNA from  
24 AATB15.2, AATA5.20 and AATA31; lane M HaeIII digested  
25  $\phi$ X174 DNA marker track]. The RNase protection assay  
26 confirmed that the beta-lactoglobulin transcription  
27 start site was being used as predicted in the mammary  
28 tissue of line AATB17 and in the salivary tissue of  
29 line AATB26. The absence of AATB transcripts in the  
30 liver of AATB17.20 and in the liver and mammary gland  
31 of AATB26.1 were also confirmed by RNase protection  
32 assays.

33

1 Protein analysis of milk  
2 Milk samples from 8 G1 females and from 5 GO females  
3 were assayed for the presence of human  
4  $\alpha_1$ -antitrypsin by the immunoblotting methods  
5 described in Example 1D. The results of this analysis  
6 are summarised in Table 3. A representative immunoblot  
7 of diluted milk samples from transgenic and normal mice  
8 is shown as Figure 13 [lanes 1, pooled human serum; 2,  
9 control mouse milk; 3, AATB 15.10 milk; 4, AATB 17.24  
10 milk; 5, AATB 17.23 milk; 6, AATB 15.20 milk; 7,  
11 control mouse milk; 8 & 9, marker proteins]. The human  
12 AAT (arrowed) is clearly evident in preparations from  
13 mice AATB17.23 and AATB17.24 and just about visible in  
14 milk from mouse AATB15.10]. Cross reaction of the  
15 anti-human sera to endogenous mouse AAT (which migrates  
16 slightly faster than its human counterpart) is also  
17 evident.

18  
19 Amounts of human  $\alpha_1$ -antitrypsin in transgenic mouse  
20 milk were estimated using LC-Partigen radial  
21 immunodiffusion plates [RID] [Behring Diagnostics,  
22 Hoescht UK Ltd, 50 Salisbury Road, Hounslow, Middlesex  
23 TW4 6JH] as described in Example 1D (see Table 3).  
24 Normal mouse milk samples with and without human  
25  $\alpha_1$ -antitrypsin were included as controls.

26  
27  
28  
29  
30  
31  
32  
33

1 TABLE 3

2	3	4	5	6
	Animal	Generation	Immunoblot	RID
	ID		presence/absence	protein mg/ml
6	AATB15.10	G1	+	NT
7	AATB15.20	G1	-	NT
8	AATB17.23	G1	+	0.448
9	AATB17.24	G1	+	0.533
10	AATB26.14	G1	-	NT
11	AATB26.28	G1	-	NT
12	AATB28.11	G1	-	NT
13	AATB28.14	G1	-	NT
14	AATB44	GO	+	0.87
15	AATB45	GO	+	0.088
16	AATB65	GO	+	0.091
17	AATB69	GO	+	0.465
18	AATB105	GO	-	-

19

20 [NT = not tested]

21

22 Of the nine different AATB transgenic mice or

23 mouse-lines examined, five efficiently directed

24 expression of human  $\alpha_1$ -antitrypsin in milk. A

25 sixth line (AATB15) also exhibited mammary expression,

26 but at lower levels. This analysis proves that the

27 AATB construct contains sufficient information to

28 direct efficient expression of human  $\alpha_1$ -antitrypsin

29 in the mammary glands of transgenic mice. There

30 appears to be some relaxation of the tissue-specificity

31 of the BLG promoter such as to allow it to function in

32 salivary gland as well as in the mammary gland. The

33 first intron of the AAT gene is not necessary for

1 efficient expression of the hybrid gene AATB. The  
2 introns and 3' flanking sequences of the BLG gene are  
3 evidently not essential for efficient mammary gland  
4 expression from the BLG promoter. The 5' flanking  
5 sequences of the BLG gene from SalI through SphI to the  
6 PvuII site in the 5'-untranslated of the BLG gene are  
7 sufficient to direct the efficient mammary expression  
8 of a heterologous gene as exemplified by AAT.

9

10 EXAMPLE 3 : COMPARATIVE EXPRESSION OF BLG CONSTRUCTS

11

12 The efficient expression of a human plasma protein in  
13 the milk of transgenic mice is exemplified by construct  
14 AATB. In this section the expression analyses of  
15 different constructs encoding a human plasma protein,  
16 either FIX or AAT, are given. The details of their  
17 constructions are given in Example 1A. Expression  
18 analyses of two configurations of the BLG gene are also  
19 given and serve to further define the BLG sequences  
20 that may be required for expression in the mammary  
21 gland. Unless otherwise stated the analyses of  
22 expression are tabulated. '+' indicates expression as  
23 determined by the presence of the appropriate mRNA  
24 transcript (detected by Northern blotting) or protein  
25 (as detected by radioimmunoassay (RIA), radial-  
26 immunodiffusion (RID), Coomassie blue staining or  
27 Western blotting. '-' indicates that expression was  
28 not detected.

29

30 **FIXA:**

31

32 Construction and expression of this construct is  
33 described in detail in WO-A-8800239 (designated



1 pSSltgXS-FIX or pSSltgXS-TARG). It comprises cDNA  
2 sequences encoding human blood clotting factor IX (FIX)  
3 inserted into the first exon of the BLG gene.  
4 Transgenic sheep have been produced which carry this  
5 construct and these have been analysed for the  
6 expression of human FIX by Northern blotting of mammary  
7 RNA and radioimmunoassays of milk:-

8				
9	Sheep	Description	RNA	FIX Protein (iu*/l)
10	6LL240	GO female	+	+: 4.7 <sup>a</sup> , 8.0 <sup>b</sup>
11	6LL231	GO female	+	+: 4.0a, 4.3b
12	7R45	G1 female@	+	+: / 5.7b
13	7R39	G1 female@	+	+: / 6.4b
14				

15 [a, analysis by RIA in 1987; b, analysis in 1988;  
16 \*, 1 iu = 5 µg; @, daughters of transgenic male 6LL225]  
17

18 The human FIX protein in transgenic sheep milk has been  
19 visualised by Western blotting and also shown to have  
20 biological activity. However, the level of protein in  
21 the milk is far below that necessary for commercial  
22 exploitation.

23

24 **AATA:**

25

26 This construct comprises the cDNA encoding human AAT  
27 inserted into the first exon of the BLG gene. It is  
28 equivalent to FIXA and thus can be considered as an  
29 example of the generalised construct designated  
30 pSSltgXS-TARG as described in WO-A-8800239. It has  
31 been used to produce transgenic sheep and mice.

32

33

1	Sheep	Description	RNA	AAT Protein*
2	6LL273	GO female	-	-
3	6LL167	GO female	nd	+ (2-10 µg/ml)
4	7LL183	GO female	nd	nd
5	*protein detected and estimated by Western blotting of			
6	milk samples			
7	nd; not done			
8				
9	Western blots of milk whey samples from normal and the			
10	two transgenic sheep analysed are shown in Figure 14			
11	[lanes 1, 7LL167(AATA); 2, control sheep whey; 3, human			
12	serum pool; 4, 7LL167(AATA); 5, 6LL273(AATA); 6,			
13	control sheep whey].			
14				
15	The human AAT (arrowed) is clearly evident in milk whey			
16	samples from 6LL167 but is not present in that from			
17	6LL273 or control sheep milk. Under these conditions			
18	endogenous AAT present in sheep milk is detected by the			
19	anti-human sera and has a greater electrophoretic			
20	mobility than its human counterpart.			
21				
22	The levels of human AAT estimated to be present in the			
23	transgenic sheep milk are low and are not sufficient			
24	for commercial exploitation.			
25				
26	Expression of the AATA construct has also been studied			
27	in transgenic mice.			
28				
29				
30				
31				
32				
33				

1	Mice	Description	RNA	AAT protein*
2	AATA1.5	line segregating	-	-
3		from AATA1		
4	AATA1.8	line segregating		
5		from AATA1	+	+ (<<2 $\mu$ g/ml)
6	AATA5	mouse-line	+	+ (2-10 $\mu$ g/ml)
7	AATA31	mouse-line	-	-

8 \*AAT protein detected and estimated by Western  
9 blotting.

10

11 Western blots of TCA precipitated whey samples from  
12 normal and transgenic mice are shown in Figure 15  
13 [Lanes 1, human  $\alpha_1$ -antitrypsin antigen (Sigma); 2,  
14 human serum; 3, mouse serum; 4, AATA 1.8.1 whey; 5,  
15 AATA 1.5.10 whey; 6, human and mouse serum; 7, control  
16 mouse whey; 8, AATA 5.30 whey; 9, AATA 1 whey; 10,  
17 human serum; 11, mouse serum]. The human AAT (arrowed)  
18 is clearly evident in preparations from mouse-line  
19 AATA5 and is just about visible in mouse-line AATA1.8.  
20 Cross-reaction of the anti-human sera with endogenous  
21 mouse AAT (which migrates slightly faster than its  
22 human counterpart) is also evident.

23

24 The levels of expression observed in mouse-line AATA5  
25 are of the same order of magnitude as is observed in  
26 transgenic sheep 7LL167, and as such would not prove  
27 commercial even if obtained in a dairy animal such as a  
28 sheep.

29

30 BLG-BLG

31

32 This construct comprises the BLG cDNA inserted into  
33 exon1 of the BLG structural gene. The construct is

1 analogous to AATA and FIXA (ie pSS1tgXS-TARG) in that  
2 the complete structural gene of BLG is present as well  
3 as the cDNA insert. In this case, however, the insert  
4 is a cDNA encoding a milk protein, rather than a cDNA  
5 from a gene normally expressed in another tissue. The  
6 expression of this construct was assessed in transgenic  
7 mice.

8

9	Mice	Description	RNA	BLG protein*
10	BB4	GO female	+	+(<.005mg/ml)
11	BB5	GO female	+	+(<.005mg/ml)
12	BB19	GO female	+	+(<.005mg/ml)
13	BB47	GO female	+	+(<.005mg/ml)
14	BB55	GO female	nd	+(<.005mg/ml)

15 \*detected and estimated by Western blotting

16 nd = not determined

17

18 The construct was expressed tissue-specifically in the  
19 four mice in which RNA was analysed. In all five  
20 animals low levels of BLG were detected in the milk.  
21 These levels of BLG are far below that observed with  
22 expression of the normal structural BLG gene (eg see  
23 Example 7 in WO-A-8800239). The data show that the  
24 'A-type' construct even when encoding a natural milk  
25 protein gene such as BLG (which is known to be capable  
26 of very high levels of expression in the mammary gland)  
27 is not expressed efficiently in the mammary gland of  
28 transgenic mice. This suggests that it may be the  
29 configuration of cDNA (whether FIX, AAT or BLG) with  
30 the genomic BLG sequence (ie insertion into the first  
31 exon) which is responsible for the low levels of  
32 expression of this type of construct.

33

1    **AATD**

2

3    This construct comprises the AAT cDNA fused to 5' BLG  
4    sequences and with 3' sequences from exons 6 and 7 of  
5    BLG and the 3' flanking sequences of the BLG gene.  
6    This gene contains no introns. Its potential for  
7    expression was assessed in transgenic mice:-

8

9    Mice	Description	RNA	AAT Protein*
10   AATD12	GO female	-	-
11   AATD14	GO female	-	-
12   AATD31	GO female	-	-
13   AATD33	GO female	-	-
14   AATD9	mouse-line	-	-
15   AAT21	mouse-line	-	-
16   AATD41	mouse-line	-	-
17   AATD47	mouse-line	-	-

18   \*assessed by Western blotting

19

20   None of the transgenic mice carrying AATD expressed the  
21   transgene.

22

23   **FIXD**   This is an analogous construct to AATD and  
24   comprises the FIX cDNA sequences fused to BLG 5' and 3'  
25   sequences (including exons 6 and 7) and contains no  
26   introns. Expression was assessed in transgenic mice.

27

28

29

30

31

32

33

1	Mice	Description	RNA	FIX Protein*
2	FIXD11	GO female	-	-
3	FIXD14	GO female	-	-
4	FIXD15	GO female	-	-
5	FIXD16	GO female	-	-
6	FIXD18	GO female	-	-
7	FIXD20	mouse-line	-	-
8	FIXD23	mouse-line	-	-
9	FIXD24	mouse-line	-	-
10	FIXD26	mouse-line	-	-

11 \*assessed by Western blotting

12

13 None of the transgenic mice carrying FIXD expressed the  
14 transgene.

15

16 These data, together with those from AATD, suggest that  
17 a simple configuration of BLG 5' and 3' sequences and  
18 target cDNA sequences (ie FIX or AAT) in which no  
19 introns are present in the construct will not be  
20 expressed efficiently, if at all, in the mammary gland  
21 of a transgenic animal.

22

23 AATC

24

25 This construct comprises the AAT cDNA inserted into the  
26 second exon of BLG. It was constructed to determine  
27 whether or not inserting the target cDNA (in this case  
28 AAT) at a site distant from the promoter (ie in the  
29 second rather than in the first exon) would improve the  
30 levels of expression. Expression was assessed in  
31 transgenic mice.

32

33

1	Mice	Description	RNA	AAT Protein*
2	AATC14	GO female	-	-
3	AATC24	GO female	-	-
4	AATC25	GO female	-	-
5	AATC30	GO female	-	-
6	AATC4	mouse-line	+	-
7	AATC5	mouse-line	-	-
8	AATC27	mouse-line	-	-

9 \*assessed by Western blotting

10

11 Only one out of seven 'lines' expressed the transgene  
12 as determined by RNA; in this line no AAT protein was  
13 detected, presumably because re-initiation from the  
14 initiating ATG of the AAT sequences did not occur. In  
15 the RNA-expressing line expression appeared to occur  
16 only in the mammary gland although at low levels.  
17 These data would suggest that moving the site of  
18 insertion of the target cDNA to the second exon (and  
19 thus including intron 1 of the BLG) does not yield  
20 improved levels of expression of the target cDNA (in  
21 this case AAT).

22

#### 23 DELTA A2

24

25 This construct contains the minimum ovine BLG sequences  
26 that have so far been shown in transgenic mice to be  
27 required for efficient and tissue-specific expression  
28 of BLG in the mammary gland. It is a 5' deletion  
29 derivative of pSS1tgXS (WO-A-8800239) and has only  
30 799 bp of sequence flanking the published mRNA cap site  
31 (Ali and Clark, (1988) J. Mol. Biol. 199, 415-426).  
32 This deleted version of pSS1tgXS has been used to  
33 produce transgenic mice.

1	Mouse	Description	RNA	BLG Protein*
2	DELTA A2/1	GO female	+	+ ~2mg/ml
3	DELTA A2/28	GO female	+	+ ~3mg/ml
4	DELTA A2/38	GO female	+	+ <0.15mg/ml

5

6 Detected by Coomassie blue staining: estimated  
7 densitometrically.

8

9 The DELTA A2 constructs shows that 799 bp of 5'  
10 flanking sequences are sufficient for correct and  
11 efficient expression of BLG in the mammary gland of  
12 transgenic mice. This construct also contains the  
13 4.9kb transcription unit of BLG and 1.9kb of 3'flanking  
14 sequences. It is conceivable that important regulatory  
15 sequences for mammary expression are present in these  
16 regions. (However, note the result with AATB in which  
17 these sequences were absent and yet efficient mammary  
18 expression was obtained.)

19

#### 20 EXAMPLE 4 : PREPARATION OF FACTOR IX CONSTRUCT

21

##### 22 Strategy

23

24 The expression in transgenic sheep of a human Factor IX  
25 gene, called BLG-FIX, is disclosed in WO-A-8800239 and  
26 Clark et al (1989) (Biotechnology, 7 487-492), both of  
27 which are herein incorporated by reference, insofar as  
28 the law allows. Since this construct has been  
29 previously referred to as FIX A, this nomenclature is  
30 retained. Essentially the FIX A construct comprises  
31 the insertion of a human FIX cDNA into the first intron  
32 of the complete (ie all exons and introns present)  
33 sheep betalactoglobulin (BLG) gene. This example



1 relates to the modification of this FIX A construct to  
2 the effect that the first intron of the human genomic  
3 FIX gene has been inserted at the appropriate position,  
4 into the FIX cDNA, so that on transcription of the new  
5 gene, a primary transcript containing an intron will be  
6 produced. When this transcript is correctly spliced, a  
7 transcript will be generated, which on translation,  
8 will generate exactly the same protein as the original  
9 FIX A construct.

10

11 The construction route shown below is complicated, but  
12 the methods used are as described in Example 1. The  
13 difficulties were caused by the size of human FIX  
14 genomic DNA fragments and the requirement to develop  
15 new shuttle vectors to allow the suitable manipulation  
16 of the BLG and FIX DNA sequences.

17

18 A.

19 Aims

20 Construction of -

21

- 22 a) pUC PM - modified cloning vector.  
23 b) pUC XS - pUC PM containing BLG genomic DNA.  
24 c) pUC XS/RV - pUC XS containing a unique EcoRV  
25 restriction site in the BLG 5'  
26 untranslated region.

27

28 Details

29

- 30 i A double stranded synthetic linker DNA including  
31 in the following order the restriction sites for  
32 the enzymes EcoRI, PvuI, MluI, SalI, EcoRV, XbaI,  
33 PvuI, MluI, HindIII (see Fig 16a) was ligated into

1     EcoRI/HindIII digested, gel purified, pUC 18  
2     (Boehringer) to generate pUC PM (see Fig 16a).  
3     The insertion was checked by both restriction  
4     analysis and direct sequencing.

5  
6     ii   A SalI-XbaI fragment purified from pSSltgXS (this  
7     contains the XbaI-SalI BLG genomic fragment in  
8     pPOLY III.I (see Figure 3 of WO-A-8800239) was  
9     ligated into SalI/XbaI digested, CIP (calf  
10    intestinal phosphatase) (see Fig 16a) - treated,  
11    gel purified, pUC PM to give pUC XS. This was  
12    checked by restriction analysis.

13  
14    iii   A synthetic EcoRV linker

15  
16           (5' TCGACGCGGCCGCGATATCCATGGATCT         )  
17           (     GCTGCGCCGGCGCTATAGGTACCTAGAGATC 5')

18  
19    was ligated into the unique PvuII site of  
20    PvuII-digested pSSltgSE (see WO-A-8800239 -  
21    pSSltgSE comprises a SphI-EcoRI fragment of BLG  
22    inserted into pPOLY III.I; the PvuII site is 30  
23    bases downstream of cap site in the first exon of  
24    BLG) - see Fig 16b.

25  
26    iv    The SphI-NotI fragment containing the EcoRV linker  
27    was gel purified from pSSltgSE/RV and ligated into  
28    the SphI, NotI digested, CIP - treated, gel  
29    purified pUC XS, generating pUC XS/RV - see Fig  
30    16b.

31  
32    This was checked by restriction analysis.

33

1 B.

2 Aims

3 Construction of -

4

5 a) Clones 9-3, B6 and 9 H11 - cloning vehicles from  
6 transfer of various portions of FIX genomic DNA.

7

8 b) Clone 11-6, this comprises exons 1, 2, 3 and  
9 introns 1, 2 of FIX inserted into pUC 9.

10

11 Details

12

13 i Cosmid clone cIX2, containing part of FIX gene,  
14 was obtained from G. Brownlee (see GB-B-2125409,  
15 also P.R. Winslip, D. Phil Thesis, Oxford, and  
16 Anson et al (1988) EMBO J. 7 2795-2799).

17

18 Note In the following description - the assignment of a  
19 base number to a restriction site refers to the  
20 number of bases the site is upstream (mins sign)  
21 or downstream of the cap site in the first FIX  
22 exon. These numbers are obtained by analogy, from  
23 the published FIX sequence of Yoshitake et al  
24 (1985) Biochemistry 24 3736-3750.

25

26 ii Clone 9-3 was produced by ligating gel purified  
27 BamHI (-2032) - EcoRI (5740) fragment from cIX2  
28 into BamHI/EcoRI-digested, CIP-treated, gel  
29 purified, pUC 9 (see Fig 17).

30

31 iii Clone 9 H11 was made by ligating the gel purified  
32 HindIII (810) - HindIII (8329) fragment from cIX2  
33 into HindIII-digested, CIP-treated, gel purified  
34 pUC 9 (see Fig 17).

1    iv    Clone 9-3 was digested with BamHI and HpaI, end  
2           filled with the Klenow enzyme, and the large  
3           fragment was gel purified and ligated to produce  
4           clone B6 (see Fig 17). The net effect of this is  
5           to remove the FIX sequence between -2032 and -830.

6  
7    v    Clone 9H 11 was digested with SalI and BglII,  
8           CIP-treated and then the large fragment, now  
9           lacking the regions between the vector SalI site  
10          and the FIX BglII site (3996) was gel purified.  
11          This was ligated with the gel purified SalI  
12          (vector) - BglII (3996) fragment from clone B6, to  
13          generate clone 11-6 (see Fig 17) which contains  
14          FIX sequence -830 - -8329 (ie exons 1,2,3 introns  
15          1,2).

16  
17    C.

18    Aims

19    Construction of -

- 20  
21    a)    Clone C8 (incorporating 5' portion of FIX cDNA).  
22    b)    Clone C81.SK (incorporating 5' portion of FIX cDNA  
23           + FIX intron I).

24  
25    Details

26  
27    i    FIX A (FIX cDNA in BLG gene, called BLG FIX in  
28          Clark et al, (1989) Biotechnology 7 487-492, also  
29          see WO-A-8800239) was digested with Sph 1/Bst Y 1.  
30          The small fragment was gel purified and ligated  
31          into SphI/BamHI-digested, CIP-treated, pUC 18  
32          (Boehringer) generating clone C8 (see Fig 18) DNA  
33          was prepared by growth in a dam<sup>-</sup> E. coli host (SK  
34          383) to allow Bcl digestion.

1 Note C8 contains most of FIX cDNA and 2 out of 3 BclI  
2 sites (at positions 2 and 81 upstream of the first  
3 nucleotide of the first AUG of the FIX cDNA  
4 sequence shown in Fig 9, GB-B-2125409; these are  
5 equivalent to Bcl sites 46 (exon 1) and 6333 (exon  
6 2) of genomic DNA.

7  
8 ii C8 was digested with BclI, CIP-treated and the  
9 large fragment retained after gel purification.

10  
11 iii Clone 11-6 DNA was prepared from E. coli host SK  
12 383 (dam<sup>-</sup>) and the 6287 bp BclI fragment  
13 containing intron 1 purified and ligated with the  
14 large C8 fragment described in ii above, to  
15 generate C81 SK - see Fig 18. The Bcl junctions  
16 were sequenced to confirm reconstruction of Bcl  
17 sites.

18  
19 4.

20 Aims

21 Construction of -

- 22  
23 a) J FIX A (FIX A insert transferred to pUC PM).  
24 b) SP FIX (A cloning vehicle for transfer of intron 1  
25 to J FIX A).

26  
27 Details

28  
29 i SphI-NotI fragment from FIX A, containing FIX cDNA  
30 and flanking BLG sequence was gel purified and  
31 ligated into SphI/NotI digested, CIP-treated, gel  
32 purified pUC XS/RV to generate J FIX A (see Fig  
33 19).

1    ii    Sph-NruI fragment containing FIX cDNA from J FIX A  
2           was gel purified and ligated into SphI/EcoRV  
3           digested, CIP treated, pSP 72 (promega Biotech) to  
4           generate SP FIX (see Fig 19).

5  
6    E.

7    Aims

8    Construction of -

9

- 10    a)    b 11 - cloning vehicle containing FIX intron 1.  
11    b)    J FIX A 1 - final "minigene" construct for  
12           construction of transgenics.

13

14    Details

15

16    i     SP FIX and C81.SK digested to completion with  
17           SphI, then partially digested with Ssp 1\*. A 7.2  
18           kb fragment from C81.SK containing FIX intron 1  
19           was ligated with the CIP-treated, gel purified  
20           large fragment of SP FIX to generate clone b 11  
21           (see Fig 20) which contains the complete FIX cDNA  
22           and FIX intron 1.

23

24    ii    The SphI-NotI fragment from b11 containing the FIX  
25           sequences was gel purified and ligated into  
26           SphI/NotI digested, CIP-treated J FIX A to  
27           generate J FIX A 1 (see Fig 20).

28

29    \*Note - In SP FIX, there is a SspI site in vector which  
30           was not excised in the partially digested fragment  
31           shown. Likewise in C81.SK there are four SspI  
32           sites in the FIX intron. The 7.2K fragment  
33           contains all these four sites and in fact

1 terminates at the SspI site at position 30830 b of  
2 the genomic FIX sequence.

3

4 F.

5

6 Transgenic mice were constructed as described in  
7 Example 1B, and identified as described in Example 1C.  
8 One male and one female transgenic mice were initially  
9 identified.

10

11

12

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33

1    CLAIMS

2

3    1.    A genetic construct comprising a 5' flanking  
4    sequence from a mammalian milk protein gene and DNA  
5    coding for a heterologous protein other than the milk  
6    protein, wherein the protein-coding DNA comprises at  
7    least one, but not all, of the introns naturally  
8    occurring in a gene coding for the heterologous protein  
9    and wherein the 5'-flanking sequence is sufficient to  
10   drive expression of the heterologous protein.

11

12   2.    A construct as claimed in claim 1, wherein the  
13   milk protein gene is a beta-lactoglobulin gene.

14

15   3.    A construct as claimed in claim 2, including about  
16   800 base pairs upstream of the beta-lactoglobulin  
17   transcription start site.

18

19   4.    A construct as claimed in claim 2, including about  
20   4.2 kilobase pairs upstream of the beta-lactoglobulin  
21   transcription start site.

22

23   5.    A construct as claimed in claim 1, wherein the  
24   heterologous protein is a serine protease.

25

26   6.    A construct as claimed in claim 2, wherein the  
27   heterologous protein is a blood factor.

28

29   7.    A construct as claimed in claim 1, in which all  
30   but one of the natural introns are present.

31

32   8.    A construct as claimed in claim 1, in which only  
33   one of the natural introns are present.



1 9. A construct as claimed in claim 1 comprising a  
2 3'-sequence.

3

4 10. A method for producing a substance comprising a  
5 polypeptide, the method comprising introducing a DNA  
6 construct as claimed in claim 1 into the genome of an  
7 animal in such a way that the protein-coding DNA is  
8 expressed in a secretory gland of the animal.

9

10 11. A method as claimed in claim 10, wherein the  
11 animal is a mammal and the secretory gland is a mammary  
12 gland.

13

14 12. A vector comprising a genetic construct as claimed  
15 in claim 1.

16

17 13. A cell containing a vector as claimed in claim 12.

18

19 14. An animal cell comprising a construct as claimed  
20 in claim 1.

21

22 15. A transgenic animal comprising a genetic construct  
23 as claimed in claim 1 integrated into its genome.

24

25 16. A transgenic animal as claimed in claim 15 which  
26 is capable of transmitting the construct to its  
27 progeny.

28

29 17. A method for producing a substance comprising a  
30 polypeptide, the method comprising harvesting the  
31 substance from a transgenic animal as claimed in claim  
32 15.

33

1/21

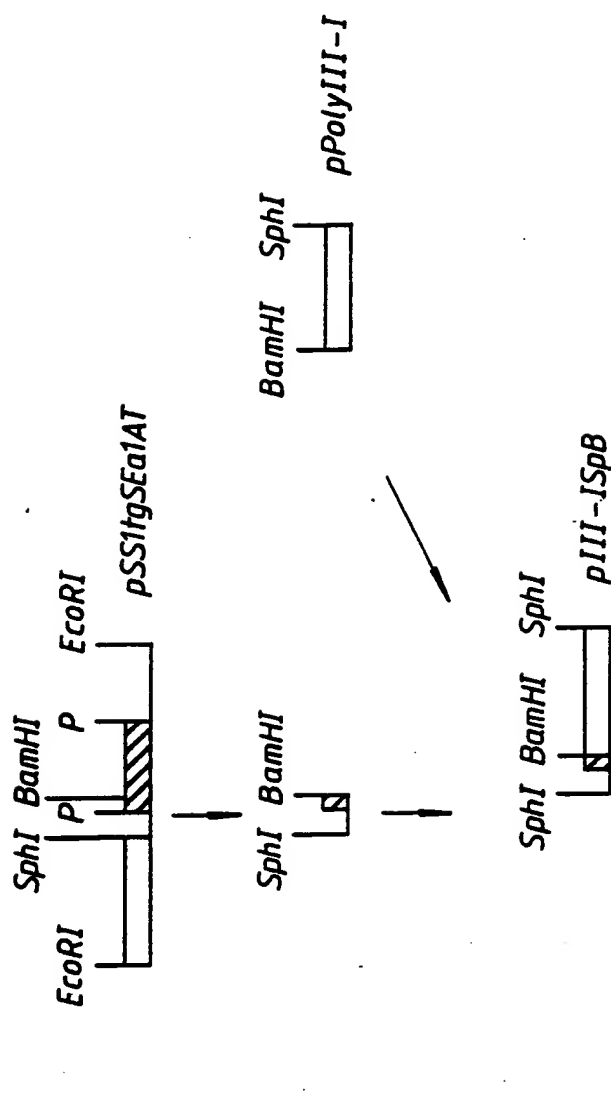


Fig.1.

2/21

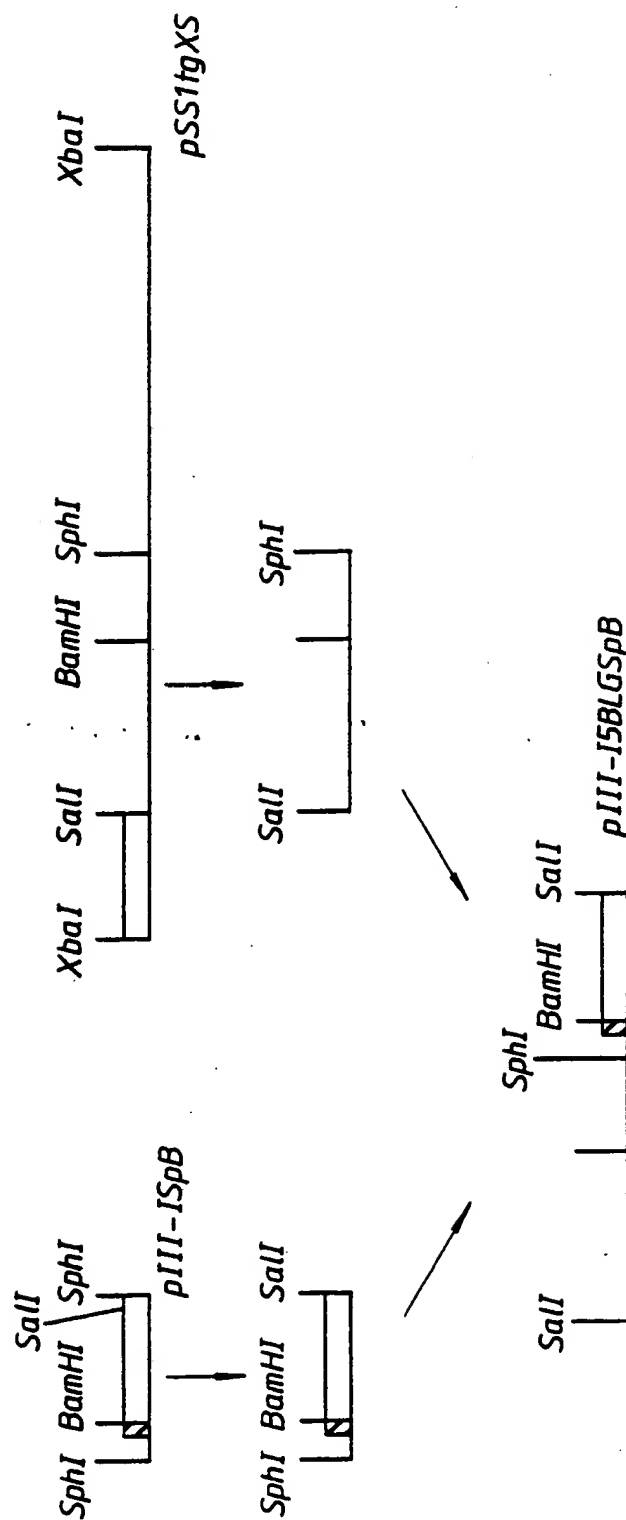
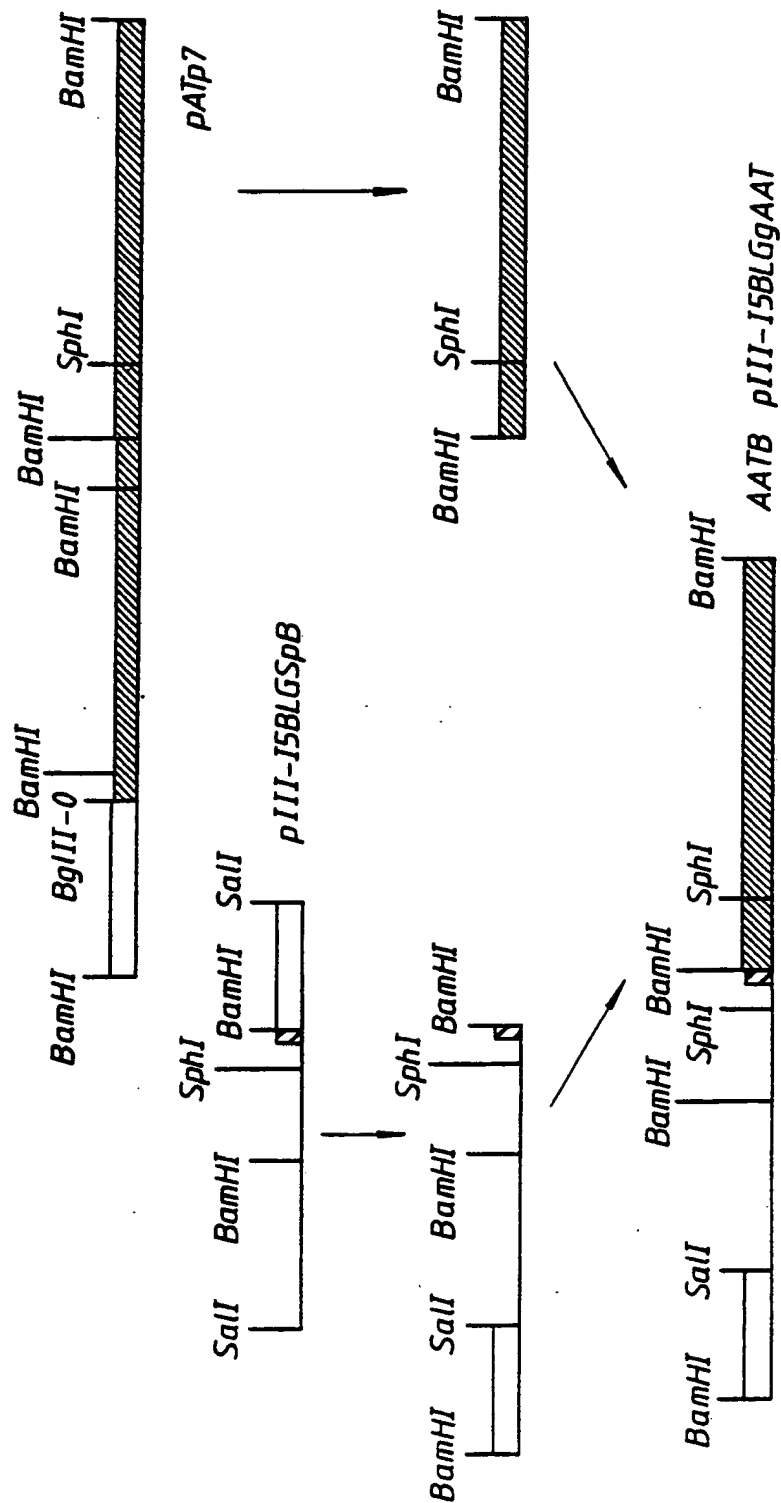


Fig.2.

3/21



alpha1-antitrypsin cDNA sequences  
alpha1-antitrypsin genomic sequences

plasmid sequences  
beta-lactoglobulin sequences

Fig.3.

4/21

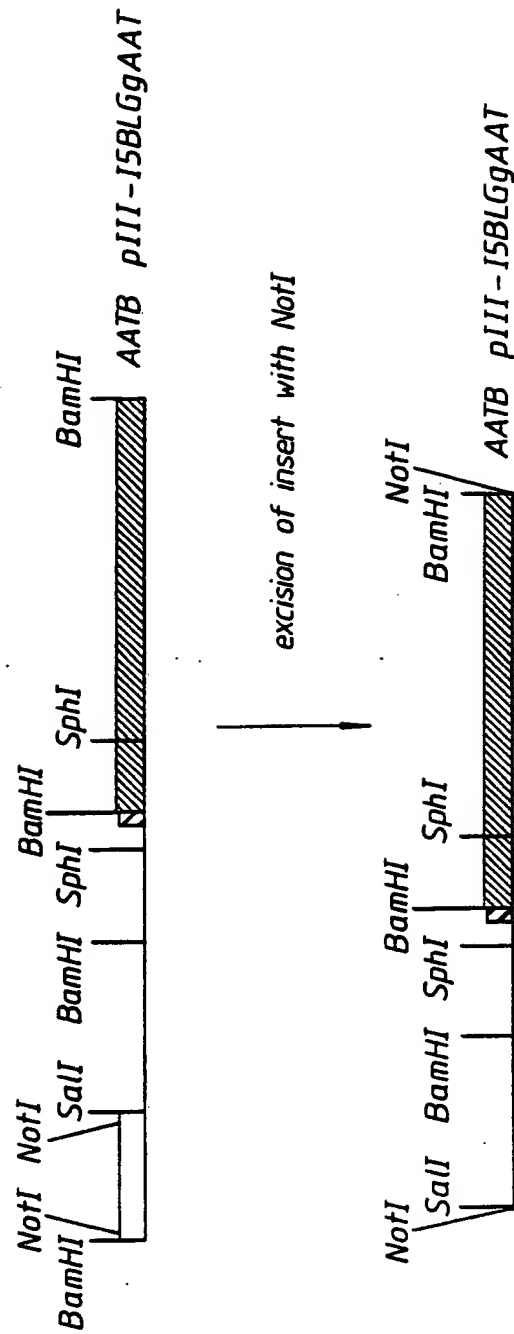


Fig.4.

5/21

SphI

gcatgcgcctcctgtataaggccccaagcctgctgtctcagccctcc

BLG | AAT

\*--&gt;

 actccctgcagagctcagaagcagacccag | cgacaatgccgtcttct  
 PvuII-0 | TaqI-0 MetProSerSer

 ValSerTrpGlyIleLeuLeuLeuAlaGlyLeuCysCysLeuValPro  
 gtctcgtggggcatcctcctgctgggcaggcctgtgctgcctgggtccct  
 ^^^

BamHI

 ValSerLeuAlaGluAspProGlnGlyAsp  
 gtctccc tggctgaggatccccaggagat

*Sequence of AATB (pIII-ISBLGgAAT) from the SphI site corresponding to the 5' flanking sequences of  $\beta$ -lactoglobulin through the fusion to the  $\alpha$ 1-antitrypsin sequences. The key restriction sites for SphI and BamHI are underlined.*

*\* = transcription start point*

*BLG =  $\beta$ -lactoglobulin*

*AAT =  $\alpha$ 1-antitrypsin*

*^^^ = indicate three nucleotides missing from the published sequence of Ciliberto, Dente & Cortese (1985) Cell 41, 531-540, but clearly present in the clone p8 $\alpha$ 1ppg procured from these authors. The nucleotides are present in the published sequence of  $\alpha$ 1-antitrypsin described by Long, Chandra, Woo, Davie & Kurachi (1984) Biochemistry 23, 4828-4837.*

Fig. 5.

6/21

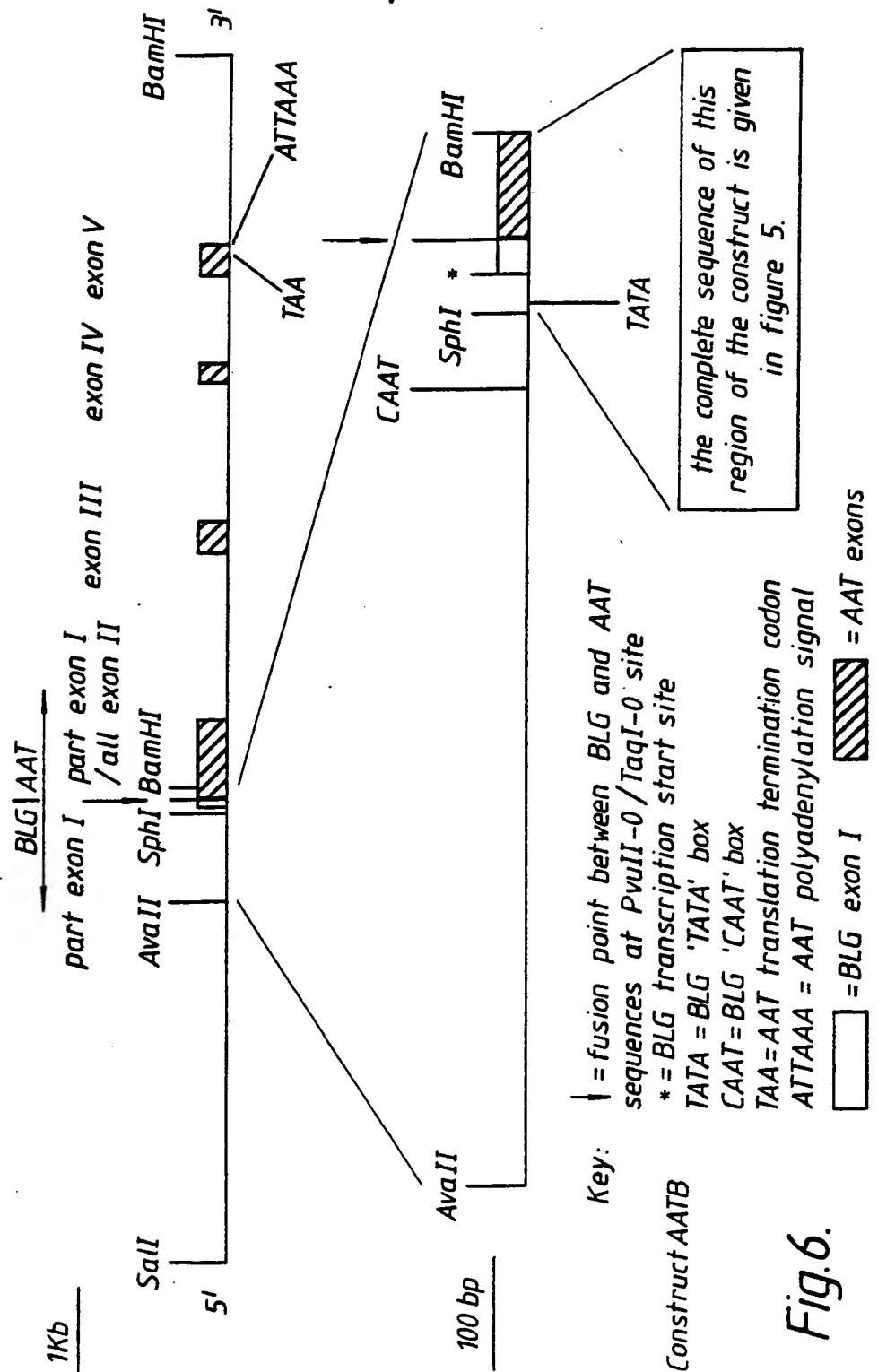


Fig.6.

7/21

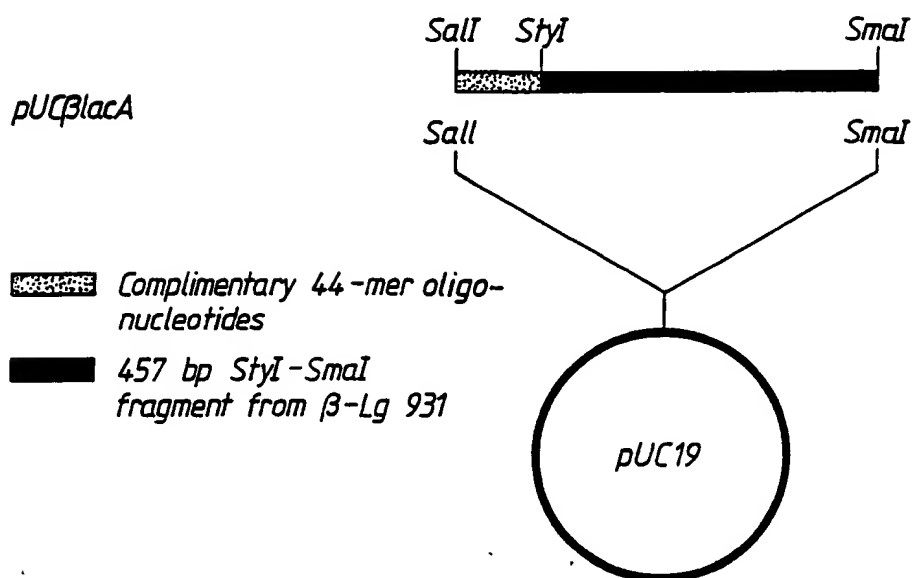
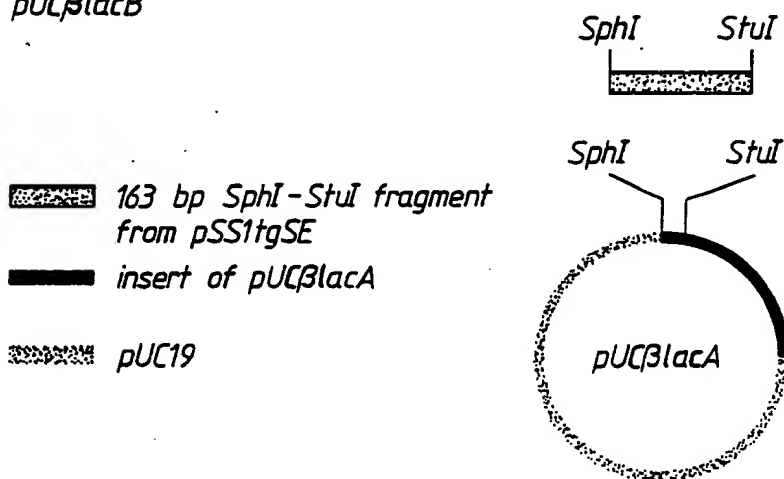
Construction of *pSS1tgXSΔC(laBLG(BB))**pUCβlacB*

Fig. 7.



8/21

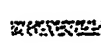
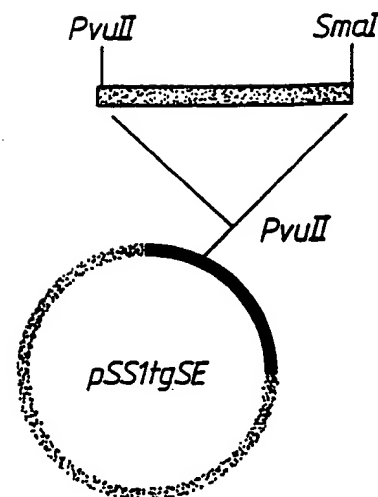
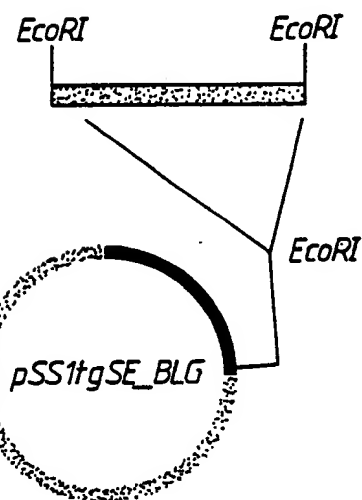

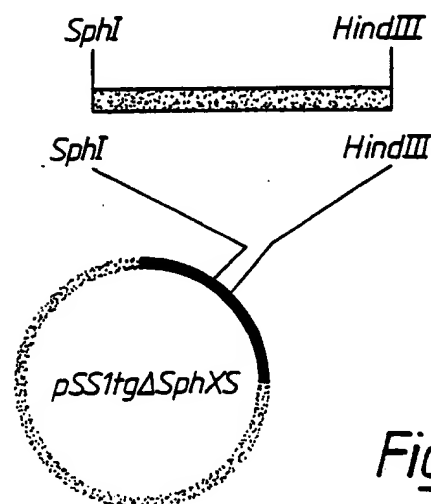
*pSS1tgSE\_BLG*
 *ppoly*
 insert of *pSS1tgSE*
*pSE\_BLG\_3'*
 5.3 *EcoRI* partial fragment from *pSS1tgXSΔCla*
 *ppoly*
 insert
*pSS1tgXSΔClaBLG*
 3 kb *SphI*-*HindIII* fragment from *pSE\_BLG\_3'*
 insert of *pSS1tgΔSphXS*
 *ppoly*


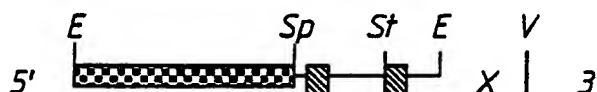
Fig. 8.

9/21

Construction of AATC:pSS1pUCXSAAT.TGA

1. Synthesis of oligonucleotides : 5' CTTGTGATATCG  
3' CACTATAGCTTAA 5'

2. Ligate annealed oligos into *StyI*/*EcoRI* cleaved pSS1tgSE to construct plasmid pSS1tgSE.TGA

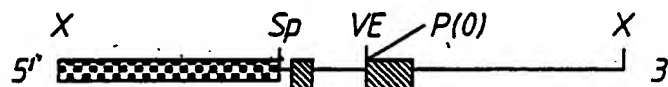


3. Cleave with *EcoRI*: Blunt with Klenow polymerase. Second cleavage with *SphI*. Isolate *SphI*-*EcoRI* (blunted) fragment.

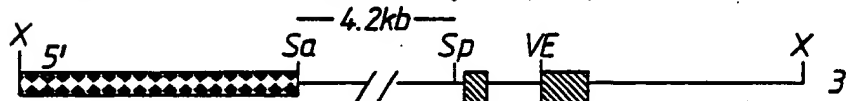


4. Cleave plasmid pBJ7 (this patent) with *SphI* and *PvuII*. Isolate large 4.3 kb) fragment.

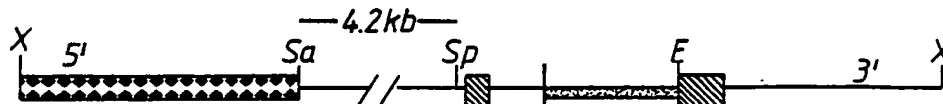
5. Ligate *SphI*-*EcoRI*(blunt) fragment (3) with *SphI*-*PvuII* fragment (4) to produce pSS1tgSpX.TGA



6. Isolate *SphI*-*XbaI* insert from pSS1tgSpX.TGA (5) and ligate to 4.2 kb *SalI*-*SphI* fragment from pSS1tgXS (previous patent) and *XbaI*-*SalI* cleaved pUC18 to yield pSS1pUCXS.TGA



7. Insert *AccI*-*HindIII* AAT insert from pUC8a1AT.73 (this patent) into the unique *EcoRV* site of pSS1pUCXS.TGA to produce pSS1pUCXSAAT.TGA. For microinjection the *XbaI*-*SalI* fragment is excised from the vector.



■ pPOLY; ■ pUC18; — BLG intron or flanking,

■ BLG exons, ■ AAT, | oligo.

E, *EcoRI*; X, *XbaI*; Sa, *SalI*; Sp, *SphI*; V, *EcoRV*; St, *StyI*; P(0), inactivated *PvuII* site.

Fig. 9.

10/21

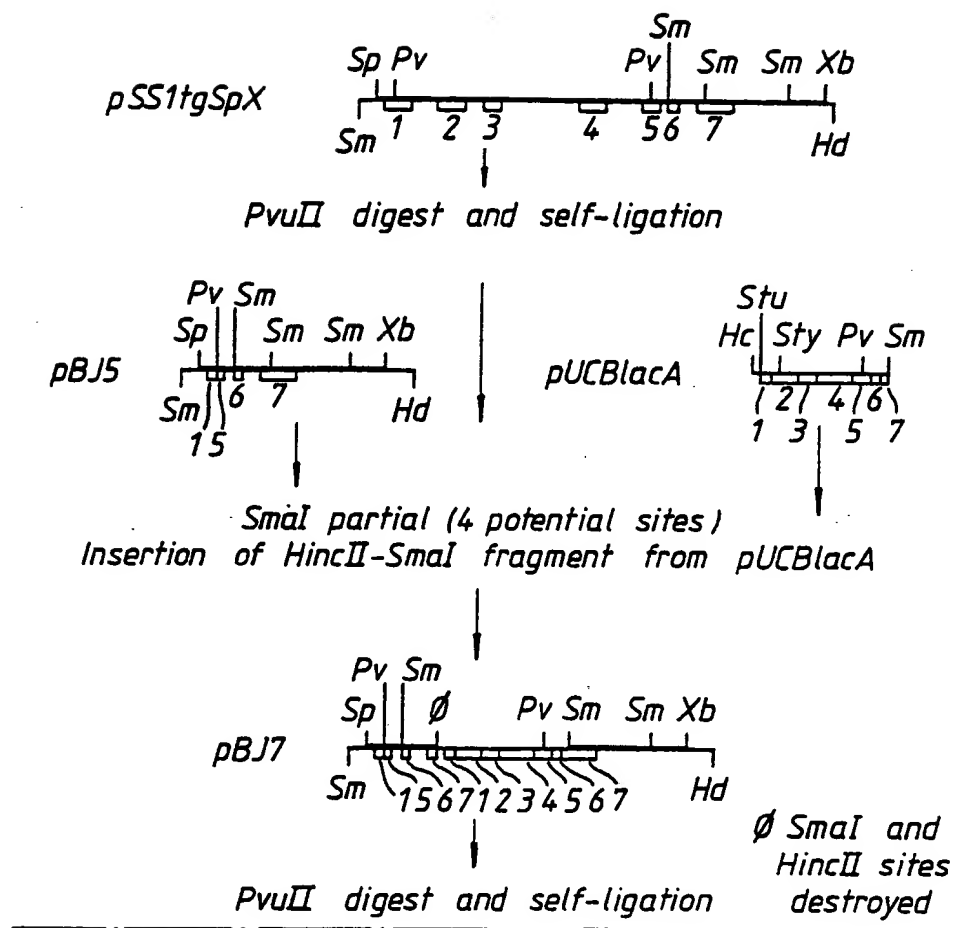


Fig.10a.

11/21

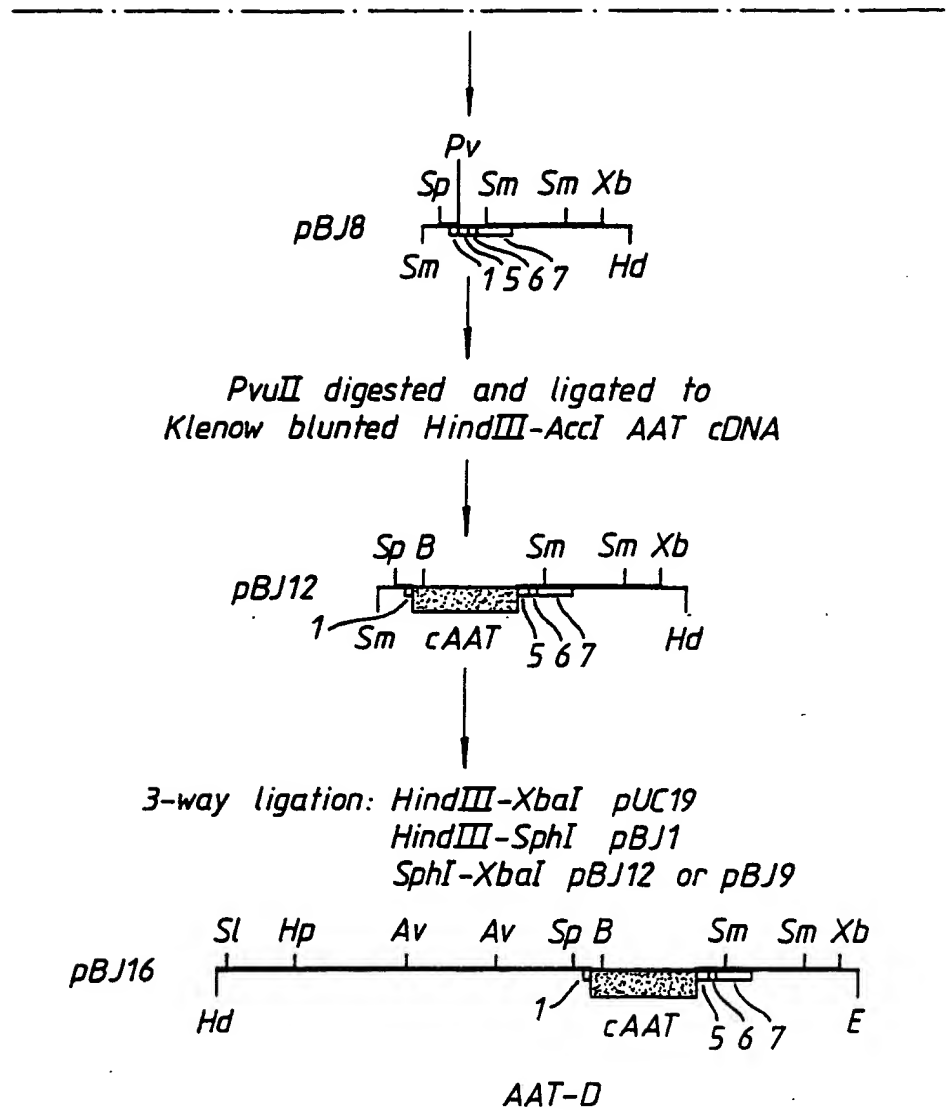
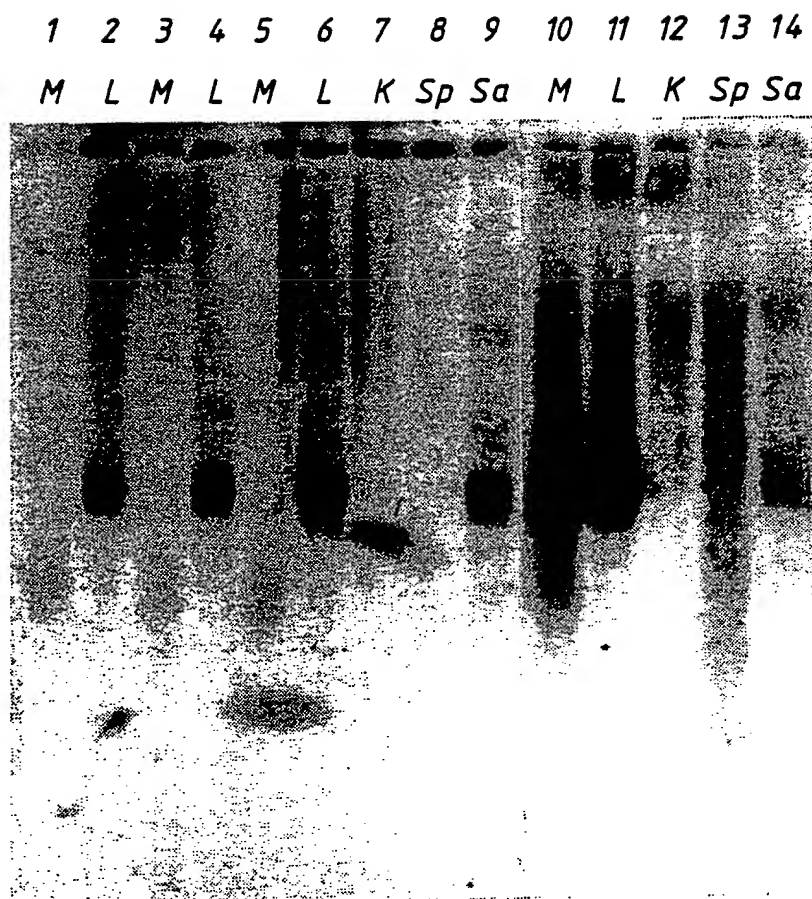


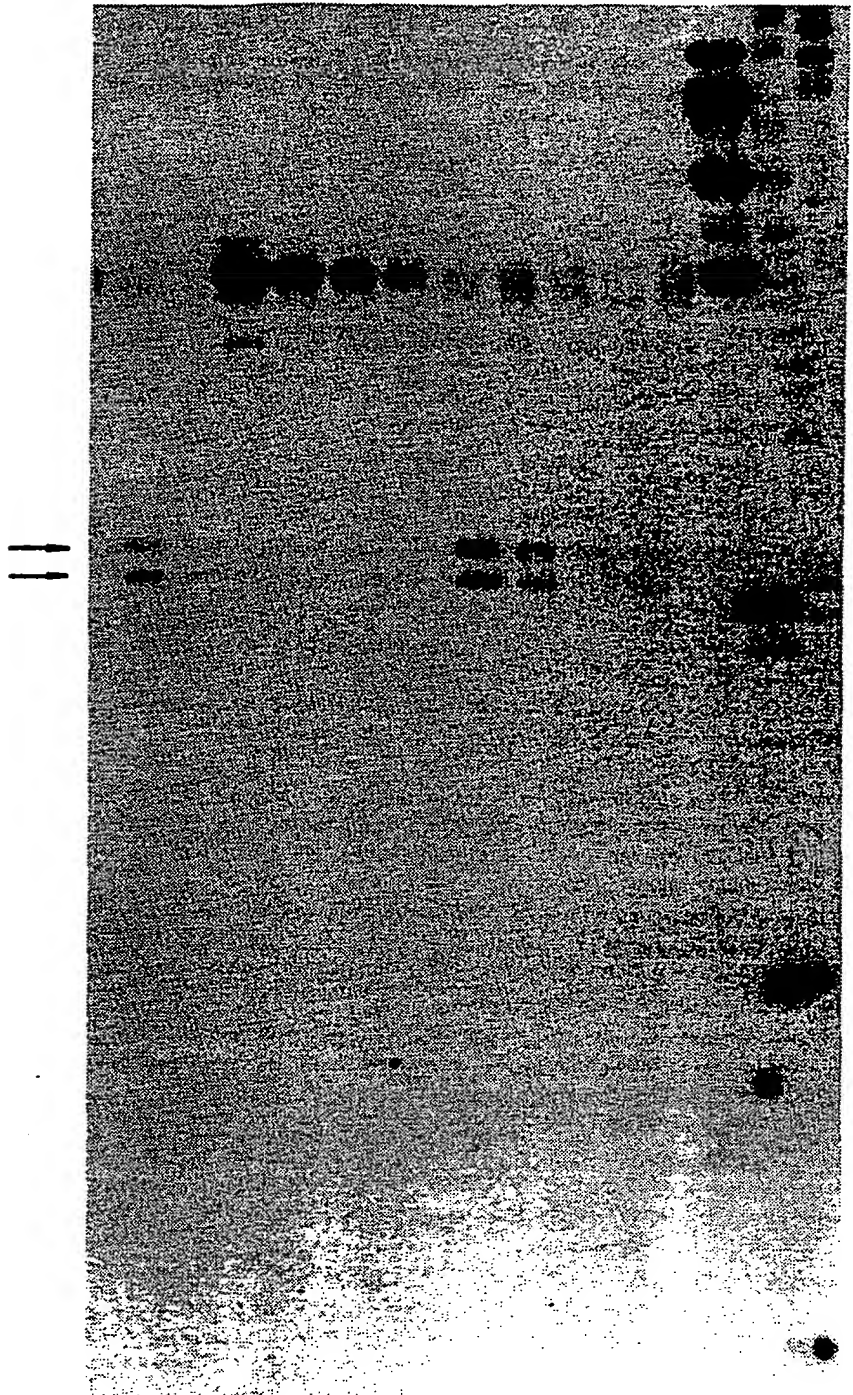
Fig.10b.

12/21

*Fig.11.*

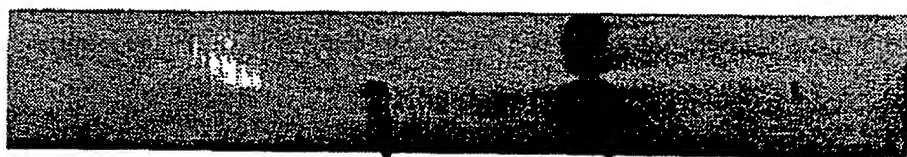
13/21

1 2 3 4 5 6 7 8 9 10 11 M

*Fig.12.*

14/21

Fig.13.



8 9



1 2 3 4 5 6 7

15/21

## EXPRESSION OF HUMAN AAT IN TRANSGENIC SHEEP MILK

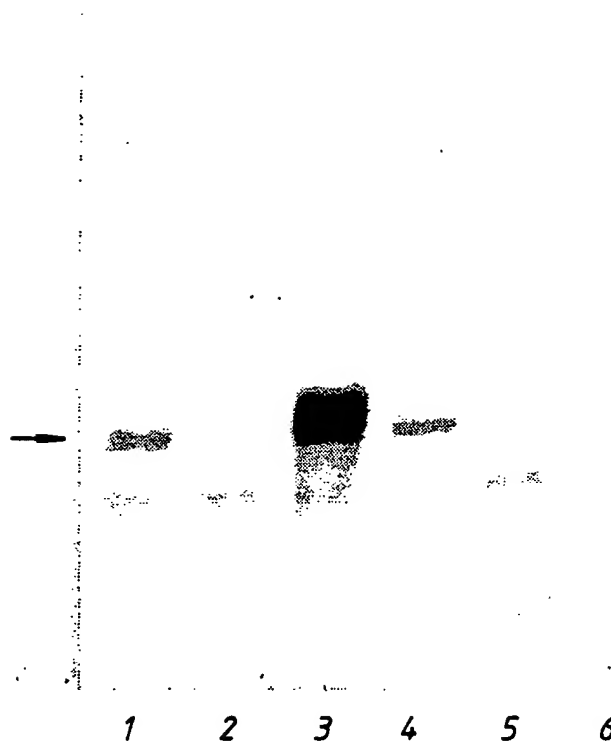


Fig.14.

## EXPRESSION OF HUMAN AAT IN THE MILK OF TRANSGENIC MICE

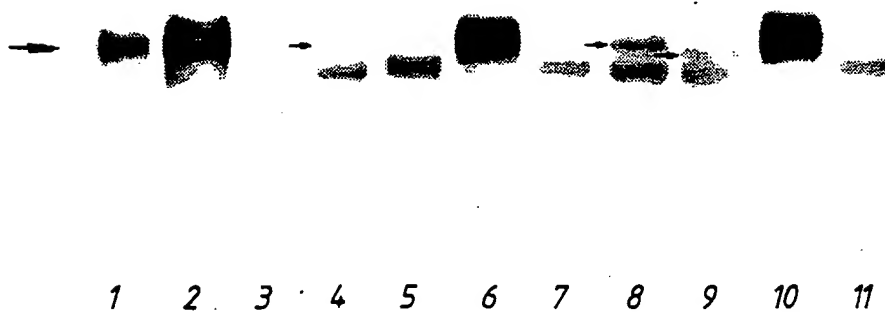


Fig.15.



16/21

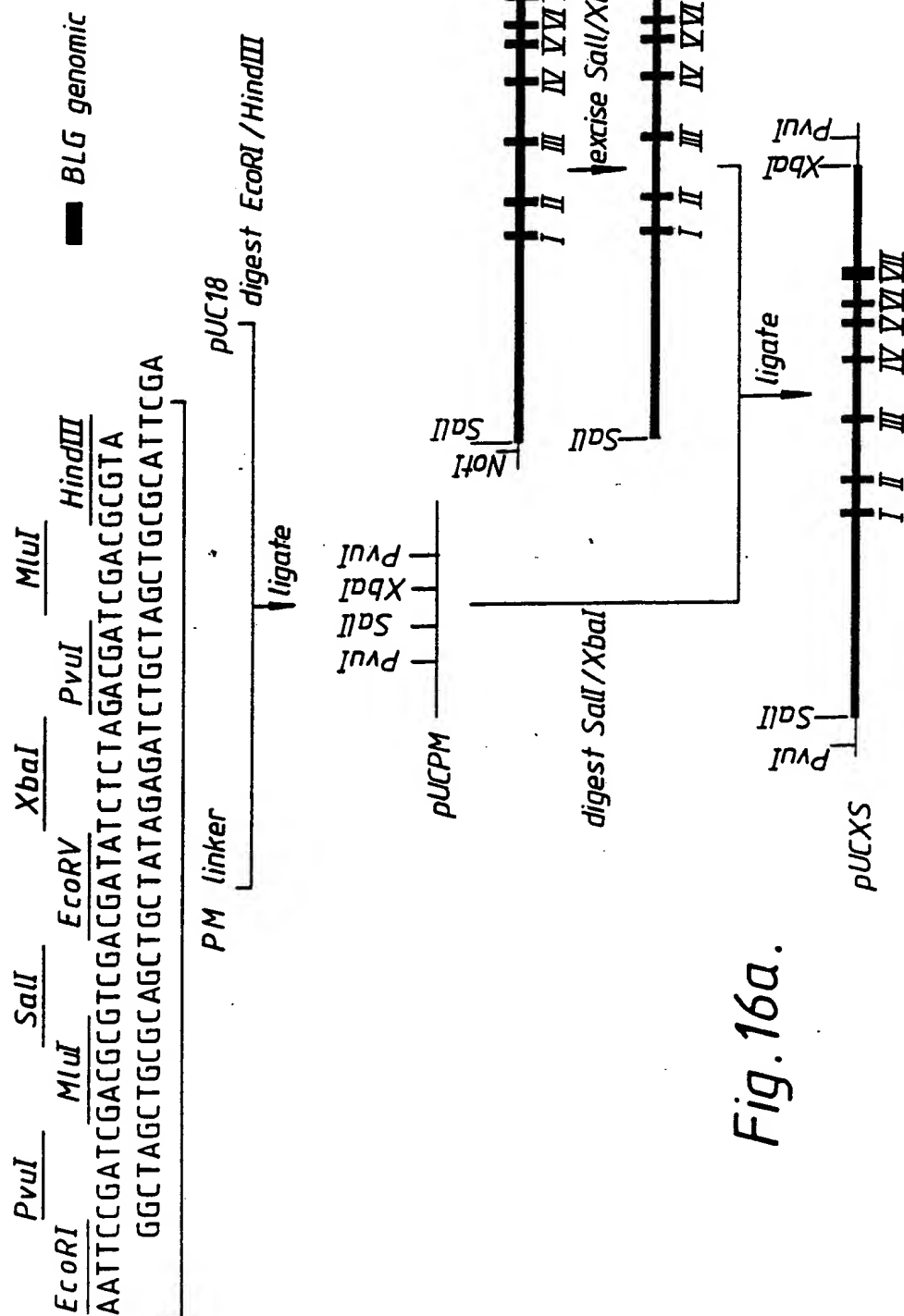


Fig. 16a.

17/21

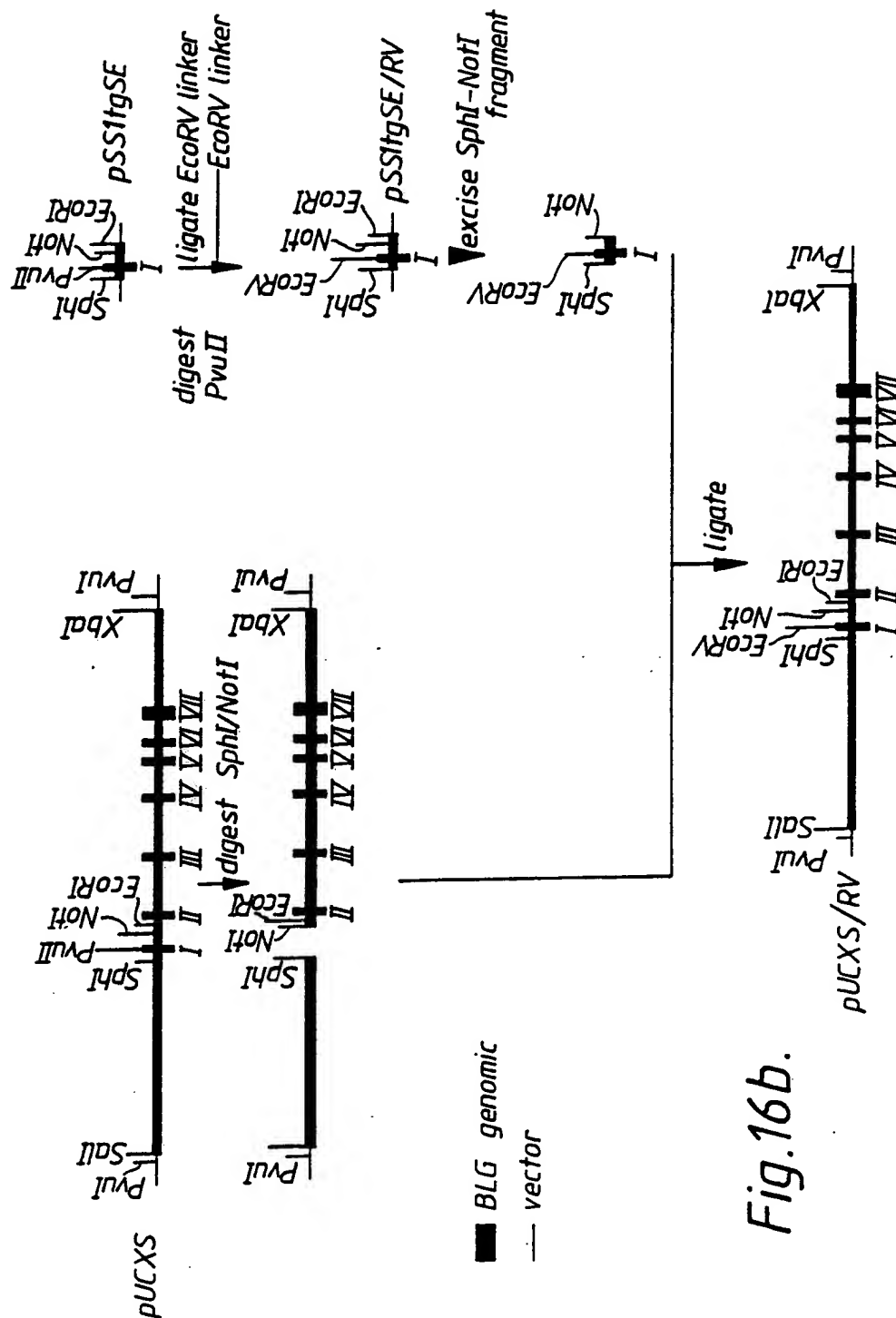


Fig. 16b.

18/21

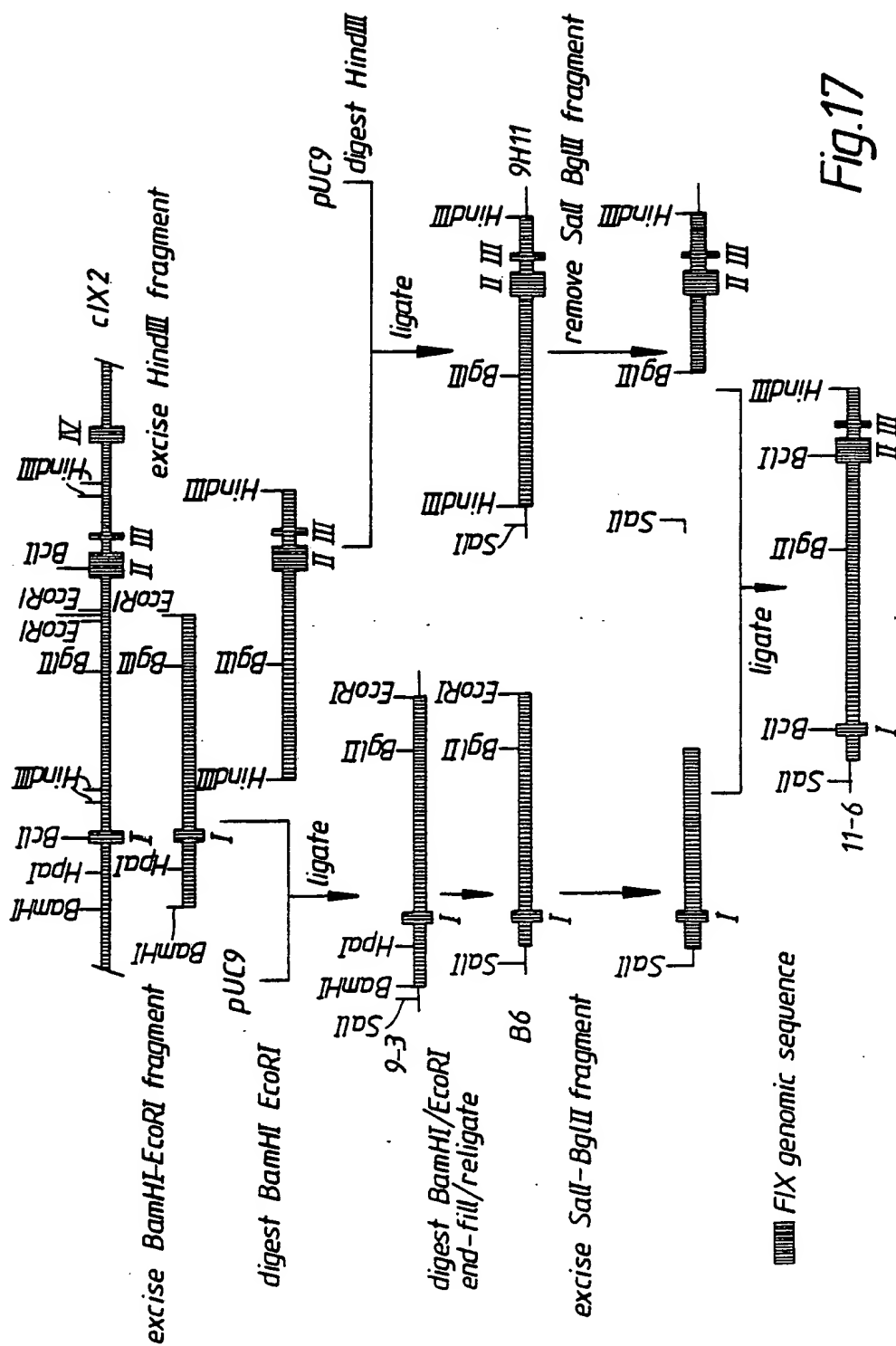


Fig.17

19/21

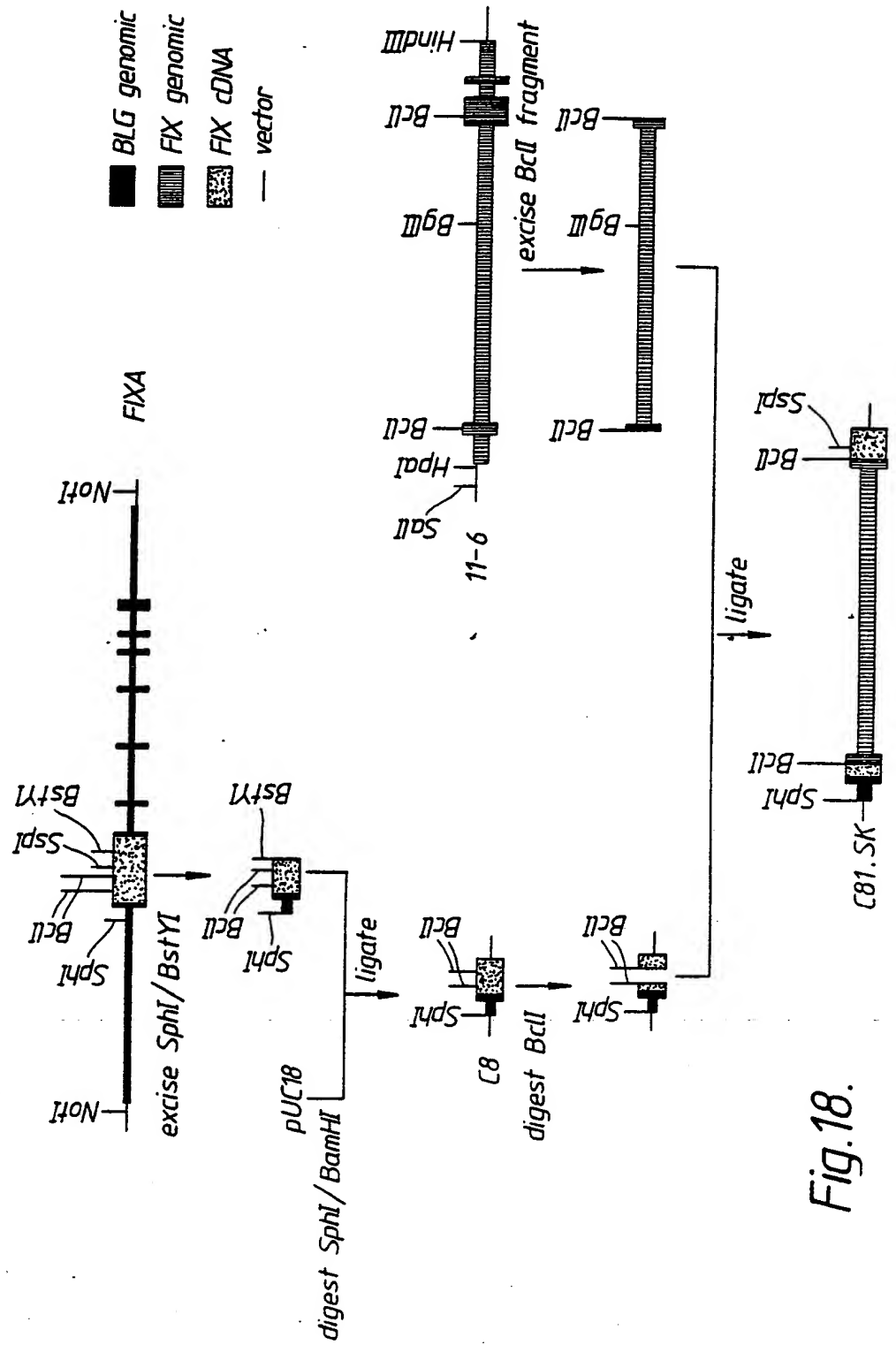


Fig.18.

20/21

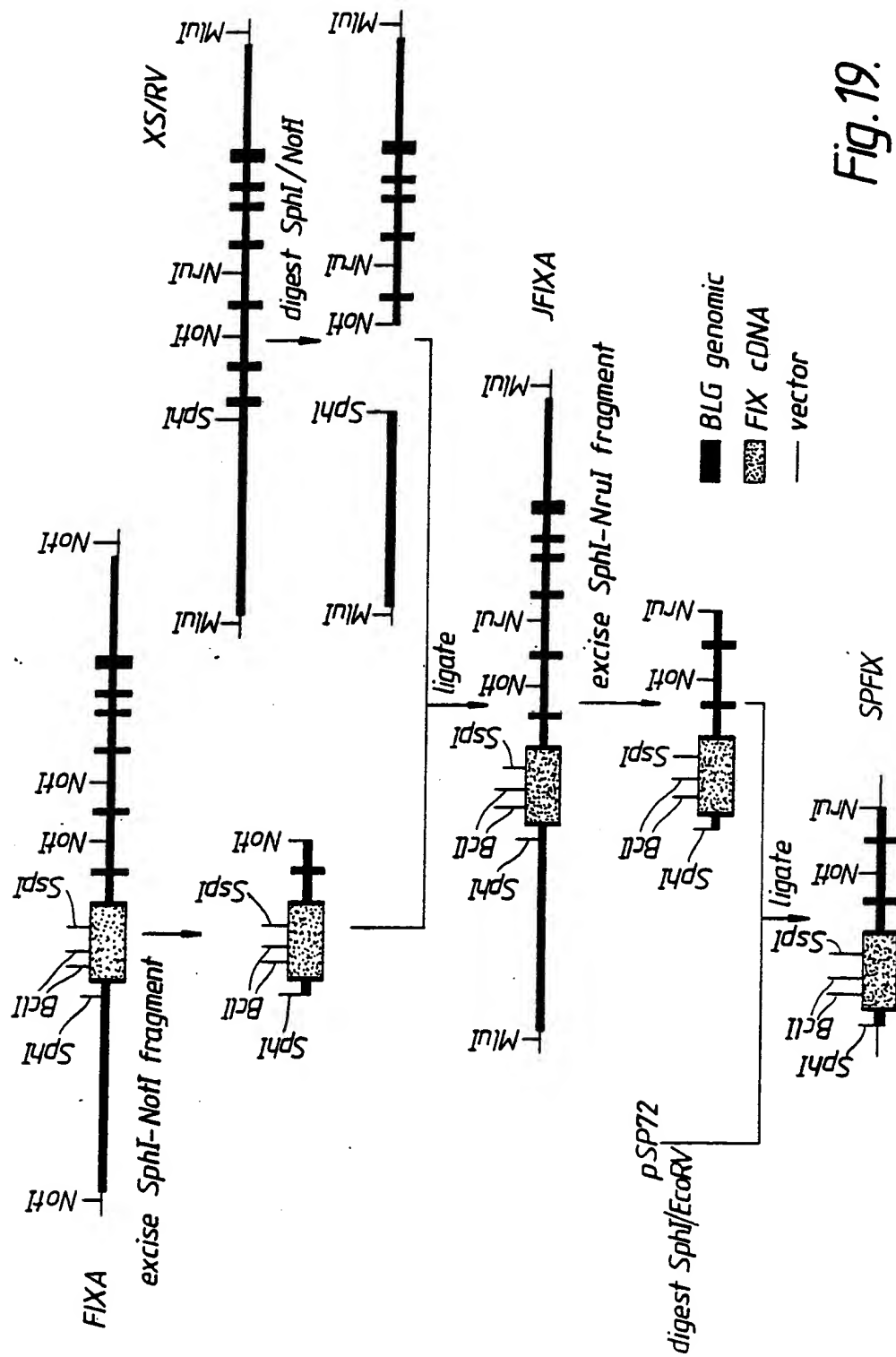


Fig. 19.



International Application No PCT/GB 89/01343

International Application No PCT/GB 89/01343

Form PCT/ISA/210 (second sheet) (January 1985)

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

P,A Chemical Abstracts, volume 110, no. 19, 8 May 1989, (Columbus, Ohio, US), Deng, Tiliang et al.: "Thymidylate synthase gene expression is stimulated by some (but not all) introns", see page 199, abstract 167168n, & Nucleic Acids Res 1989, 17 (2), 645- 58

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V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 15, 16 because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(ii)  
Plant or animal varieties or essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes.

2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims: it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.



ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 89/01343

SA 32133

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on 08/11/89  
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 88/00239	14/01/88	AU-D- 76490/87 EP-A- 0274489 JP-T- 1500162	29/01/88 20/07/88 26/01/89
EP-A1- 0264166	20/04/88	JP-A- 63000291	05/01/88

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

# ZYMOGENETICS

11

**REDACTED**

**VIA EXPRESS MAIL**

Dr. Andrew Carver  
Pharmaceutical Proteins Ltd.  
Roslin  
Edinburgh  
Midlothian  
Scotland EH25 9PP

**REDACTED**

Dear Andy:

**REDACTED**

**Novo Nordisk**



Dr. Andrew Carver  
[REDACTED]

Page Two

**REDACTED**

Please let me know who should be named as an inventor. Under U.S. law, an inventor is one who made an inventive contribution to the claimed subject matter. Although this definition sounds circular, in general an inventor is one who made an intellectual contribution to the conception or reduction to practice of the invention, that is one who contributed more than routine technical skills to solving the problem(s) addressed by the invention. Determination of inventorship can be a thorny issue, and it may be appropriate to discuss the matter over the telephone.

**REDACTED**

Sincerely,



Gary Parker  
Manager, Patent Department

GP/at

Enclosures

# Facsimile Transmission

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Attention: Gary Parker

Company: ZymoGenetics

Telephone #:

Fax #: 0101 206 632 4009

Subject: Patent

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Sender: Andy Carver

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COMMENTS: Dear Gary,

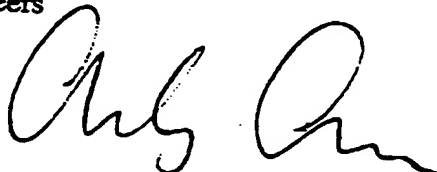
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As for the Inventors, we realise the importance of the choice with regards to patent legality and therefore suggest Ian Garner, Mike Dalrymple from PPL and Donna Prunkard and Don Foster from Zymo. It looks if you use word 5 on an IBM so why don't you e-mail me the finished document if you can ( I can BINhex 4.0!!! to decompress or maybe you can save it as a self extracting archive? ) I can easily covert it into WordPerfect on the Mac. My e-mail number is [carver@pplros.demon.co.uk](mailto:carver@pplros.demon.co.uk)

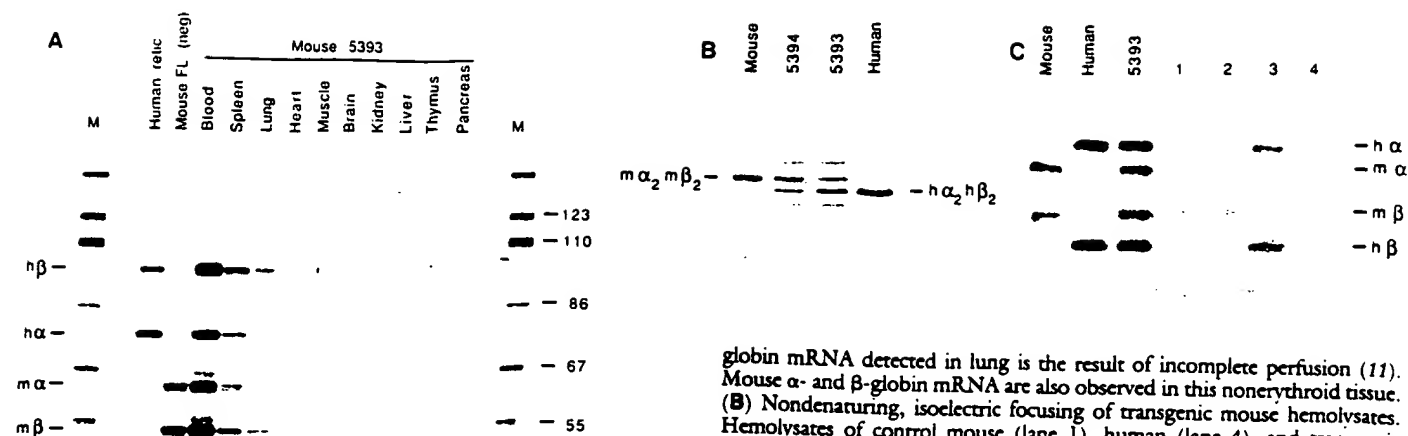
PS

Thanks for the promotion but I'm a Mr

Cheers





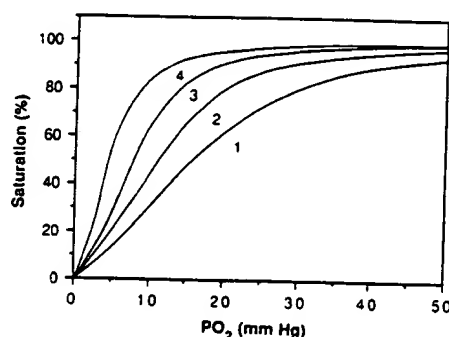


**Fig. 2.** Expression of human  $\alpha$ - and  $\beta$ -globin genes in transgenic mice. (A) Primer extension analysis of total RNA from ten tissues of an HS I,II  $\alpha$ -globin/HS I,II  $\beta$ -globin transgenic mouse. Human reticulocyte and mouse fetal liver RNAs are controls. Authentic human  $\beta$ - and  $\alpha$ -globin primer extension products are 98 bp and 76 bp, respectively; correct mouse  $\alpha$ - and  $\beta$ -globin products are 65 and 53 bp, respectively (1, 8). Human  $\alpha$ - and  $\beta$ -

globin mRNA detected in lung is the result of incomplete perfusion (11). Mouse  $\alpha$ - and  $\beta$ -globin mRNA are also observed in this nonerythroid tissue. (B) Nondenaturing, isoelectric focusing of transgenic mouse hemolysates. Hemolysates of control mouse (lane 1), human (lane 4), and transgenic mouse (lanes 2 and 3) blood were run on a native agarose isoelectric focusing gel (12) and photographed without staining. (C) Denaturing, cellulose acetate strip electrophoresis of transgenic mouse hemoglobins. Hemoglobins were denatured in alkaline-urea buffer, electrophoresed on cellulose acetate strips, and stained with imido black (13). Lanes marked mouse, human, and 5393 are hemolysates of control mouse, human, and transgenic mouse (5393) blood, respectively. Lanes marked 1 to 4 are hemoglobins purified from individual bands (numbered 1 to 4 from top to bottom) of sample 5393 on the isoelectric focusing gel in (B).

( $h\alpha_2h\beta_2$ ) and mouse hemoglobin ( $m\alpha_2m\beta_2$ ) are observed. In addition to human and mouse hemoglobins, two other major bands were observed in both transgenic samples. To determine the composition of these bands and to confirm the human and mouse hemoglobins, the four bands in sample 5393 were excised from the gel and analyzed on a denaturing cellulose acetate strip (13) (Fig. 2C). Control lysates of mouse, human, and 5393 blood samples were separated in lanes on the left. Mouse  $\alpha$ - and  $\beta$ -globin polypeptides, as well as human  $\alpha$ - and  $\beta$ -globin polypeptides, were well separated on this strip. Sample 5393 contained all four polypeptides; the human  $\alpha$ - and  $\beta$ -globin polypeptides were 110% and 106% of the amounts of mouse  $\alpha$ - and  $\beta$ -globin, by densitometric analysis. The top band (band 1) of sample 5393 in Fig. 2B is composed of human  $\alpha$ - and mouse  $\beta$ -globin chains. The second band is mouse  $\alpha$ - and mouse  $\beta$ -globin and the third band is human  $\alpha$ - and  $\beta$ -globin as expected. The polypeptides composing band 4 in Fig. 2B are barely visible in Fig. 2C but are clearly mouse  $\alpha$ - and human  $\beta$ -globin. Therefore, normal amounts of human hemoglobin can be synthesized in adult mice, and multiple combinations of globin polypeptides are possible [see note (14)].

The functional properties of human, mouse, and hybrid hemoglobins synthesized by transgenic mice were assessed by determination of oxygen equilibrium curves (OEC) and by calculation of  $P_{50}$  values. The  $P_{50}$  is the partial pressure at which hemoglobin is half saturated with oxygen and is inversely related to hemoglobin oxygen affinity. All four hemoglobins described above were purified by preparative IEF (15) and



**Fig. 3.** Oxygen equilibrium curves (OEC) of hemoglobins purified from 5393 transgenic mouse progeny. Hemoglobins of 5393 progeny were separated on preparative isoelectric focusing gels (15). Bands 1 to 4 (top to bottom) illustrated in Fig. 2B were purified from gel slices and the OEC of each hemoglobin band was determined in 0.1M potassium phosphate, pH 7.0 at 20°C (16). The  $P_{50}$  of band 1 ( $h\alpha_2m\beta_2$ ) is 15.7 mmHg, band 2 ( $m\alpha_2m\beta_2$ ) is 11.1 mmHg, band 3 ( $h\alpha_2h\beta_2$ ) is 8.0 mmHg, and band 4 ( $m\alpha_2h\beta_2$ ) is 4.7 mmHg. The  $P_{50}$  of human hemoglobin in these transgenic mice is identical to the  $P_{50}$  of native Hb A.

the OEC for each was determined (16) (Fig. 3). The OEC were normal, sigmoid-shaped, and demonstrate that all four hemoglobins bind oxygen cooperatively. The  $P_{50}$  of human hemoglobin synthesized by transgenic mice is 8.0 mmHg, which is identical to the  $P_{50}$  of native human Hb A. Interestingly, the oxygen affinities of the two hybrid tetramers differ significantly from human and mouse hemoglobins. The  $h\alpha_2m\beta_2$  hybrid has an extremely low O<sub>2</sub> affinity; the  $P_{50}$  is 15.7 mmHg. In contrast, the O<sub>2</sub> affinity for  $m\alpha_2h\beta_2$  is extremely high; the  $P_{50}$  for this hemoglobin is 4.7 mmHg (17).

Finally, the hematological values of six

transgenic progeny were determined and compared to five normal animals. Red blood cell counts and hematocrits for transgenic animals were normal and, interestingly, the values for hemoglobin and mean corpuscular volume were in the normal range. Consequently, the calculated values of mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration (MCHC) for transgenic animals were normal. Thus the total hemoglobin concentration in transgenic erythrocytes is not increased even though reticulocytes contain 100% more globin mRNA (18). Therefore, to maintain normal MCHC, all globin mRNAs are either translated at reduced rates or  $\alpha$ - and  $\beta$ -globin polypeptides are less stable. Another possibility is that globin synthesis ceases when the maximum intracellular concentration of hemoglobin is attained. If the rate of globin synthesis is normal, then a full complement of hemoglobin could be synthesized in half the time leading to faster maturation of reticulocytes.

In summary, the results presented demonstrate that high levels of human  $\alpha$ - and  $\beta$ -globin mRNA can be coexpressed in mice. The transgenes are expressed specifically in erythroid tissue and levels of human hemoglobin equivalent to mouse hemoglobin can be achieved. In addition, the human hemoglobin produced in these mice is fully functional and the transgenic animals are phenotypically normal. These results provide a solid foundation for the production of transgenic mice that synthesize high levels of other human hemoglobins. We have initiated studies to synthesize high levels of human sickle hemoglobin in transgenic mice in an attempt to produce a mouse model of sickle

cell disease. Although sickle cell anemia was the first disease to be understood at the molecular level (19), there is still no cure or adequate treatment. If a transgenic mouse model can be developed, new drug therapies and even gene therapies could be tested. Once perfected in model systems, protocols that are safe and effective for humans could be developed.

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10. Adult animals were made anemic with phenylhydrazine (20) to induce reticulocytosis, anesthetized, perfused (11), and tissues were removed. Total RNA was prepared from frozen tissue [*Anal. Biochem.* 162, 156 (1987)] with the following modification. The final RNA pellets were resuspended in a solution containing 1.0% SDS, proteinase K (100 mg/ml), 25 mM NaCl, 1.0 mM EDTA, and 10 mM tris-HCl pH 7.5. After digestion for 3 hours at 50°C, the samples were extracted with phenol/chloroform, chloroform, and ethanol precipitated.
11. Animals were perfused by cutting the right atrium and injecting phosphate-buffered saline into the left ventricle. The lung is not perfused in this procedure and, therefore, is contaminated with blood.
12. Blood cells were washed twice with saline and lysed in a volume of water equal to the cell pellet. One-fourth volume of carbon tetrachloride was mixed with the hemolysate, and cell membranes were extracted by brief vortexing and microcentrifugation. The aqueous phase was removed and frozen at 20°C. Samples were subsequently thawed, diluted with an equal volume of 0.05% KCN, and separated on an agarose isoelectric focusing gel (Resolve-Hb, Isolabs Inc., Akron, Ohio) according to the manufacturer's specifications. After focusing, proteins were fixed in the gel with 10% trichloroacetic acid for 10 min. The gel was then rinsed for 1 hour with water, dried, and hemoglobin bands were visualized without staining.
13. Hemoglobin bands were cut out of the agarose IEF gel and eluted in water for 1 hour at room temperature. After dialysing against water overnight at room temperature, the samples were lyophilized and resuspended in water. Equal volumes of sample (purified hemoglobin or whole hemolysate), alkaline-urea buffer (6.0M urea, 15 mM boric acid, 0.5 mM EDTA, 25 mM tris-HCl, pH 8.6), and  $\beta$ -mercaptoethanol were mixed and an aliquot was loaded onto a cellulose acetate strip (Gelman) that had been soaked overnight in alkaline-urea buffer. The samples were then electrophoresed for 1 hour at 190 V in alkaline-urea buffer. Proteins were subsequently stained with 0.5% imido black in methanol:acetic acid (45:10). The strips were destained in methanol:acetic acid (47.5:5), dried, and photographed.
14. Although only four hemoglobin bands are observed on the IEF gel in Fig. 2B, nine hemoglobins representing all possible combinations of mouse and human  $\alpha$ - and  $\beta$ -globin polypeptides probably exist inside the cell. During electrophoresis oxy-hemoglobin tetramers ( $\alpha_2\beta_2$ ) dissociate into dimers ( $\alpha_1\beta_1$ ) that are separated by charge differences. Therefore, hemoglobin tetramers composed of dimers of unlike charge are not detected (21).
15. Mouse, human, and hybrid hemoglobins synthesized by transgenic mice were separated by preparative IEF on 4.0% acrylamide gels containing 2.0% Pharmalyte pH 5 to 8. Each of the four bands was sliced from the gel, homogenized, and the hemoglobin was eluted in 0.1M potassium phosphate buffer. The isolated fractions were concentrated with Amicon filters (YM 10).
16. Hemoglobins were maintained in the carbon monoxide (CO) form during separation and concentration procedures to avoid auto-oxidation. Prior to functional studies the hemoglobins were converted to the oxy-state by photolysis and vacuum removal of CO. The oxygen equilibrium curve of each hemoglobin fraction was determined using a Hemox Analyzer (TCS, Southampton, PA) in 0.1M potassium phosphate buffer, pH 7.0 at 20°C (22). All samples were analyzed four times and the curves were drawn in continuous mode. The maximum error of measurement of the  $P_{50}$  values is  $\pm 1$  mmHg [*Crit. Care Med.* 7, 391 (1979)].
17. OEC of whole blood and unfractionated hemolysates from transgenic mice were also determined and compared to mouse and human controls. The curve for whole blood of 5393 transgenic progeny is virtually identical to the mouse control, while the curve for an unfractionated transgenic hemolysate is shifted to the left of the mouse hemolysate control. The left shift of the transgenic hemolysate OEC can be attributed to the presence of high-affinity hybrid and human hemoglobin species. The similarity of the whole blood OEC for transgenic and control mice may be due to adaptive responses, such as an increase in allosteric effectors of oxygen affinity, in the transgenic mice.
18. Quantitative solution hybridizations of blood RNA from the seven transgenic lines indicate that mouse  $\alpha$ - and  $\beta$ -globin mRNA levels (picograms of total RNA per microgram) are not decreased in mice expressing high levels of human  $\alpha$ - and  $\beta$ -globin mRNA.
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23. We thank N. Martin, J. Askins, and M. Avarbock for excellent technical assistance, J. Prchal for providing human reticulocyte RNA, K. Hall for instructions on electrophoresis of hemoglobins on denaturing cellulose acetate strips, and J. Engler for synthesizing the human  $\alpha$ -, human  $\beta$ -, mouse  $\alpha$ -, and mouse  $\beta$ -globin oligonucleotides. Supported in part by grants HL-35559, HD-09172, HL-38632, and HD-23657 from the National Institutes of Health and predoctoral training grant T32 CA-09467 from NIH (to T.R.).

13 April 1989; accepted 26 July 1989

## Circumsporozoite Protein Heterogeneity in the Human Malaria Parasite *Plasmodium vivax*

RONALD ROSENBERG,\* ROBERT A. WIRTZ, DAVID E. LANAR, JETSUMON SATTABONGKOT, TED HALL, ANDREW P. WATERS, CHUSAK PRASITTISUK

Phenotypic heterogeneity in the repetitive portion of a human malaria circumsporozoite (CS) protein, a major target of candidate vaccines, has been found. Over 14% of clinical cases of uncomplicated *Plasmodium vivax* malaria at two sites in western Thailand produced sporozoites immunologically distinct from previously characterized examples of the species. Monoclonal antibodies to the CS protein of other *P. vivax* isolates and to other species of human and simian malarias did not bind to these nonreactive sporozoites, nor did antibodies from monkeys immunized with a candidate vaccine made from the repeat portion of a New World CS protein. The section of the CS protein gene between the conserved regions I and II of a nonreactive isolate contained a nonapeptide repeat, Ala-Asn-Gly-Ala-Gly-Asn-Gln-Pro-Gly, identical at only three amino acid positions with published nonapeptide sequences. This heterogeneity implies that a *P. vivax* vaccine based on the CS protein repeat of one isolate will not be universally protective.

**M**ALARIA, A DISEASE CAUSED BY A mosquito-borne protozoan parasite of red blood cells, is so widespread and causes disability so severe that many strategies to control it have been devised. Recently much effort has been focused on the construction of vaccines designed to elicit a host immune response to sporozoites, the parasite stage injected into

humans by mosquitoes. The predominant surface, or circumsporozoite (CS), proteins of sporozoites are characterized by tandemly repeated peptide units that occupy about one-third of each molecule and are immunogenic (1-3). In *Plasmodium vivax*, one of four *Plasmodium* species naturally infecting humans, the unit has been found to be a nonapeptide repeated about 20 times (2, 3).

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903 This Week in *Science*

## Editorial

905 AAAS Presidents

## Letters

908 Monitoring the U.S. AIDS Epidemic: R. BERKELMAN, J. CURRAN, W. DARROW, T. DONDERO, M. MORGAN; E. O. LAUMANN, J. H. GAGNON, S. MICHAELS ■ NRDC on Alar: R. M. WHYATT

## Association Affairs

915 Science Education in the United States: What the Scientific Community Can Do: W. E. MASSEY

## News & Comment

923 The Cystic Fibrosis Gene Is Found ■ The CF Gene Hits the News  
926 FAA's Bomb Scanner: An Awkward Goliath?  
927 NSF's Summer of Discontent

## Research News

928 Triton Steals Voyager's Last Show ■ Why Neptunian Ring Sausages?  
931 Superconductor Patents: Four Groups Duke It Out ■ A Costly Lesson in Patent Law  
933 A Surprise Near Virgo  
934 Briefings: LEP Comes from Behind ■ How to Stop Tearing Your Hair Out ■ One Giant Crash for Mankind ■ Chinese Science Students Wooed ■ Psychiatrists Get Treatment Manual

## Articles

939 Drug Prohibition in the United States: Costs, Consequences, and Alternatives: E. A. NADELMANN  
947 Organization of the Human Brain: M. S. GAZZANIGA

## Research Articles

952 Enhancement of Bacteriophage T4 Late Transcription by Components of the T4 DNA Replication Apparatus: D. R. HERENDEEN, G. A. KASSAVETIS, J. BARRY, B. M. ALBERTS, E. P. GEIDUSCHEK

## Reports

959 Geological and Geochemical Record of 3400-Million-Year-Old Terrestrial Meteorite Impacts: D. R. LOWE, G. R. BYERLY, F. ASARO, F. J. KYTE  
962 Silicon Coordination and Speciation Changes in a Silicate Liquid at High Pressures: X. XUE, J. F. STEBBINS, M. KANZAKI, R. G. TRØNNES

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**COVER** An embryonic grasshopper jumping leg imaged in a laser confocal scanning microscope, computer-enhanced, and pseudo-colored. The developing nervous system is labeled with fluorescent, neuron-selective antibodies. A major leg sensory nerve (far right) has failed to connect to the central nervous system because of the absence of a single pair of pioneer neurons. See page 982. [Photograph by Monika Klose, David Bentley, and Janet Duerr]

- 965 A Protein That Binds to a cis-Acting Element of Wheat Histone Genes Has a Leucine Zipper Motif: T. TABATA, H. TAKASE, S. TAKAYAMA, K. MIKAMI, A. NAKATSUKA, T. KAWATA, T. NAKAYAMA, M. IWABUCHI
- 967 Recognition of Thymine-Adenine Base Pairs by Guanine in a Pyrimidine Triple Helix Motif: L. C. GRIFFIN AND P. B. DERVAN
- 971 Synthesis of Functional Human Hemoglobin in Transgenic Mice: R. R. BEHRINGER, T. M. RYAN, M. P. REILLY, T. ASAKURA, R. D. PALMITER, R. L. BRINSTER, T. M. TOWNES
- 973 Circumsporozoite Protein Heterogeneity in the Human Malaria Parasite *Plasmodium vivax*: R. ROSENBERG, R. A. WIRTZ, D. E. LANAR, J. SATTABONGKOT, T. HALL, A. P. WATERS, C. PRASITTSUK
- 976 Fitness Differences Among Remnant Populations of the Endangered Sonoran Topminnow: J. M. QUATTRO AND R. C. VRIJENHOEK
- 978 Subplate Neurons Pioneer the First Axon Pathway from the Cerebral Cortex: S. K. MCCONNELL, A. GHOSH, C. J. SHATZ
- 982 Transient Pioneer Neurons Are Essential for Formation of an Embryonic Peripheral Nerve: M. KLOSE AND D. BENTLEY
- 984 Mapping Neuronal Inputs to REM Sleep Induction Sites with Carbachol-Fluorescent Microspheres: J. J. QUATTROCHI, A. N. MAMELAK, R. D. MADISON, J. D. MACKLIS, J. A. HOBSON

## AAAS Meetings

- 987 1990 AAAS Annual Meeting ■ Natural Sciences & Technology ■ Global Change ■ Social & Behavioral Sciences ■ Advance Registration and Housing Form

## Book Reviews

- 991 The Evolution of Technology, reviewed by: C. A. DUNLAVY ■ Chemical Mediation of Coevolution, D. J. FUTUYMA ■ Digging into the Past, J. T. GREGORY ■ The Fractional Quantum Hall Effect, R. JOYNT ■ Immunoglobulin Genes, W. DUNNICK ■ Books Received

## Products & Materials

- 996 Direct-Reading Emission Spectrometer ■ Fume Hood Needs No Outside Vent ■ Photochemical Reactor Enhances HPLC Detection ■ Coating for Custom Microscope Slides ■ Advanced Electronic Stirrers ■ Ultrafiltration System ■ Peptide Mapping System for Liquid Chromatography ■ Literature

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